REMARKS

Claims 1-11 and 18 and 19 are pending in the application. Claims 12-17 and 20 are withdrawn from consideration. For the Examiner's convenience, Applicants' remarks are presented in the order in which they were raised in the Office Action. Reconsideration is respectfully requested.

Rejections and Objections of Specification

The Examiner noted that the trademarks "PERKIN ELMER" and "TITERTEK TWINREADER" should be capitalized and accompanied by the generic terminology. The specification has been amended to indicate the proprietary nature of the trademarks. Applicants have also amended the trademark "GENBANK" to indicate the proprietary nature.

The Examiner objected to the embedded hyperlink and/or other form of browser-executable code. The hyperlink and/or form of browser executable code has been removed from the specification.

No new matter is added.

Claim Rejections under 35 USC § 103

(i) Claims 1, 3-6, 9, 18 and 19 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Potempa et al. in view of Nakayama et al. and Bramanti et al.

Potempa is cited for teaching a HA-2 containing gingipain from P. gingivalis. The Examiner cites to a 1705 amino acid protein from P. gingivalis with a protiona having a 99.25% homology to SEQ ID NO:5. It is acknowledged that Potempa does not teach an antagonist of an interaction between a P. gingivalis molecule and a HA2-binding motif on a porphyrin molecule.

Nakayama is cited for teaching that P. gingivalis binds heme. It is acknowledged that Nakayama does not teach an antagonist of an interaction between a P. gingivalis molecule and a HA2-binding motif on a porphyrin molecule.

Bramanti is cited for teaching agents that antagonize the interaction between a P. gingivalis molecule and hemin (Bramanti Table 2).

Applicants submit that while Bramanti shows a specific interaction between Omp26 protein and hemin, it does not characterize OMP26 as a HA2 protein and does not characterize any interactions between a HA2 domain and hemin. Bramanti does not provide the sequence of OMP26 and therefore it would be impossible to know from Bramanti which portions or combinations of portions of the Omp26 protein are involved in the reported interactions. The polyclonal antibodies that puportedly link their study to OMP26 were not characterized in terms of specificity to any particular domain(s) of Omp 26 in Bramanti (Bramanti Fig. 3).

Subsequent publications state that: "sequence analysis and trypsin susceptibility [shows that] the hemin-binding Omp26 described by Bramanti and Holt is clearly different from the HA2 domain." (DeCarlo AA, Paramaesvaran M, Yun PL, Collyer C, Hunter N. *Porphyrin-mediated binding to hemoglobin by the HA2 domain of cysteine proteinases (gingipains) and hemagglutinins from the periodontal pathogen Porphyromonas gingivalis.* J Bacteriol. 1999 Jun;181(12):3784-3791, at 3790, left col., second full paragraph). OMP26 could not even be sequenced by Bramanti due to N-terminal blockage. (Kim SJ, Chu L, Holt SC. *Isolation and characterization of a hemin-binding cell envelope protein from Porphyromonas gingivalis.* Microb. Pathog. 1996 Jul; 21(1):65-70, at 68). Bramanti does not teach or suggest a HA2 protein. While OMP26 displays functional similarities with an HA2 domain, they are different proteins. No functional information about the HA2 polypeptide is shown in the Bramanti reference.

A conclusion of obviousness is proper "so long as it takes into account only knowledge which was within the level of ordinary skill in the art at the time the claimed invention was made and does not include knowledge gleaned only from applicant's disclosure." *In re McLaughlin* 443 F.2d 1392, 1395, 170 USPQ 209, 212 (CCPA 1971). The present invention, for the first time,

determines "the molecular mechanism of HA2 domain binding to porphyrin-containing molecules such as hemoglobin and in particular heme." (Specification, page 4, lines 13-15) Thus, Applicants submit that the citation of the combination of Potempa et al., Nakayama et al. and Bramanti et al. as obviating the present claims is based on improper hindsight reasoning, since none of the references teach the role of a HA2 domain of a protein in porphyrin binding nor was any such role known to one of skill in the art at the time of these publications.

Neither Potempa et al., Nakayama et al. or Bramanti et al., by themselves or in combination, teach or suggest "administering an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a molecule derived from said microorganism having an HA2 domain and an HA2-binding motif on a porphyrin containing molecule" as specified in independent claim 1 (and independent claim 9) of the present application. Claims 3-6, 18 and 19 depend from claim 1. Therefore, Applicants respectfully request withdrawal of this ground for rejection.

(ii) Claim 11 stands rejected under 35 U.S.C. § 103(a) as being unpatentable over Progulske-Fox et al. (WO 96/17936) in view of Nakayama et al. and Bramanti et al.

Progulske-Fox is cited for teaching a method for using a hagA gene and an HA2-containing molecule containing the sequence of a useful marker for HA2. ("amino acid sequence defined by <400>1 [SEQ ID NO:1] ... is not the porphyrin binding site but a useful marker for HA2." Specification, page 24, lines10-11). Applicants submit that Progulske-Fox does not teach or suggest any function of a HA2 domain of a protein in porphyrin binding.

As discussed above, neither Nakayama nor Bramanti teach or suggest a role for HA2 in porphyrin binding. Since, Progulske-Fox, Nakayama and Bramanti, by themselves or in combination, teach or suggest a method "to antagonize the interaction between a *P. gingivalis*-derived HA2-containing molecule ... and an HA2-binding motif " as specified in independent claim 11, Applicants respectfully request withdrawal of this ground for rejection.

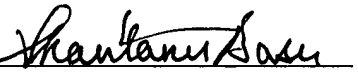
CONCLUSION

In light of the amendments and arguments set forth above, Applicants earnestly believe that they are entitled to a letters patent and respectfully request the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 229752001500. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: May 7, 2004

Respectfully submitted,

By 

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Microbial Pathogenesis 1996; **21**: 65–70

Short communication

Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*

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Kim, S.-J. (Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284, U.S.A.), L. Chu and S. C. Holt. Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*. *Microbial Pathogenesis* 1996; **21**, 65–70.

A 30 kDa (heated 24 kDa) hemin-binding protein whose expression is both hemin and iron regulated was identified and purified in *Porphyromonas gingivalis* 381. A strong hemin-binding function was found by LDS-PAGE and TMBZ staining when cells were grown under hemin (iron)-limited conditions. N-terminal amino acid sequence analysis of CNBr-digested 24 kDa hemin binding protein revealed that this protein belongs to a new, so far undescribed hemin-binding class of proteins.

Key words: Hemin; *Porphyromonas gingivalis*; TMBZ; outer membranes.

Introduction

While there are several studies which have dealt with the expression of hemin-regulated proteins in the oral pathogen, *Porphyromonas gingivalis*,[1–5] there are few studies which have demonstrated actual hemin-binding. Smalley *et al.*[5] identified a major tetramethylbenzidine(TMBZ) staining 32 kDa (unheated) protein in hemin-limited *P. gingivalis* W50, while Grenier[6] demonstrated that the hemin-binding property of *P. gingivalis* ATCC 33277 is mediated by the lipopolysaccharides, particularly the Lipid A region. In the study presented here, we have identified, purified, and characterized a putative hemin binding protein in *P. gingivalis* 381.

Results

Identification of hemin-binding protein

In Fig. 1(a), the upregulation of the 56 and 30 kDa proteins (unheated) is very clear. TMBZ staining [Fig. 1(b)] revealed the only protein to bind the stain was at 30 kDa when cells were grown under hemin(iron) restriction. Note in Fig. 1(b) that

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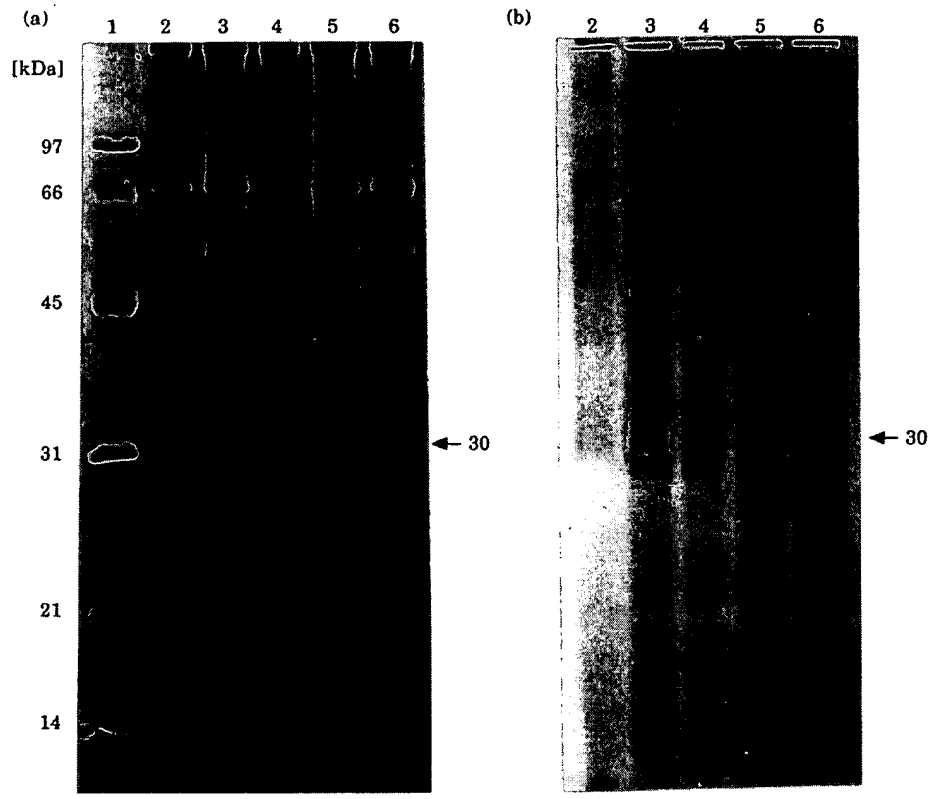

Fig. 1. Coomassie Brilliant blue stained (a) and tetramethylbenzidine (TMBZ) stained (b) LDS-PAGE of cell envelopes from *P. gingivalis* 381. Protein (70 μg) was applied to each lane following incubation with hemin. Lane 1, low mol.wt. standards; lane 2, cells grown + 7.7 μM hemin; lane 3 passage 5; lane 4, cells grown + 200 μM BPD; lane 5, cells grown + 300 μM BPD; lane 6, cells grown + 400 μM BPD.

with increasing iron restriction (i.e. 300, 400 μM BPD), there was increased TMBZ binding. A series of very lightly staining TMBZ bands might correspond to LPS.

Purification of hemin-binding protein

The 30 kDa *P. gingivalis* 381 hemin-binding protein was sequentially purified from the unheated cell envelopes (Fig. 2). In Fig. 2, lane 2, the cell envelope fraction contained numerous cell envelope proteins, including LPS-associated proteins. 1% CHAPS solubilization resulted in the removal of a large number of membrane proteins and associated LPS. The 30 kDa protein in Fig. 2, lane 3 was the major protein. The 30 kDa protein was isolated from the SDS-PAGE gels of the 1% CHAPS-soluble fraction (Fig. 2, lane 4). This protein was aggregated with proteins at 24 and 56 kDa. Heating of the isolated 30 kDa protein resulted in the appearance of the 24 kDa protein and several other minor and weakly staining proteins (Fig. 2, lane 5). The 24 kDa protein was isolated from the SDS-PAGE of this heated 30 kDa protein (Fig 2, lane 6). The resulting 24 kDa protein was isolated from the gel as a single protein band, with no contaminating proteins even when the gels were overloaded with large amounts of purified protein (data not shown).

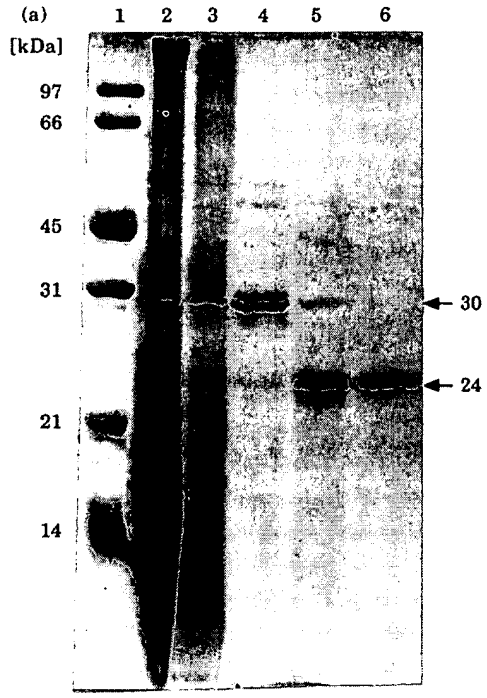

Fig. 2. SDS-PAGE analysis of the purification of the 30 kDa (unheated; 24 kDa heated) hemin-binding protein from *P. gingivalis* strain 381. Lane 1, low mol.wt. standards; lane 2, cell envelope fraction; lane 3, 1% CHAPS-soluble fraction; lane 4, isolation of 30 kDa protein; lane 5, 100°C, heated isolation 30 kDa protein; lane 6, purified 24 kDa protein.

Table 1 Cyanogen bromide fragmentation of 24 kDa hemin-binding protein from *Porphyromonas gingivalis* 381

Fragments	Amino acid sequence*
20 kDa	DQATSVPTDG(X)Y(X)TVD(X)KLGRITVK
17 kDa	GPDG(H)ZMEYEE
12 kDa	EYEEQGFSEVITGKKNAQGFAA(X)G(X)LEF(S)

*(X), unknown; (), assume to be correct amino acid.

N-terminal sequence analysis

The CNBr digestion of 24 kDa hemin-binding protein revealed at least three polypeptide bands (data not shown). Internal amino acid sequence analysis of one of these fragments (12 kDa) is seen in Table 1.

Discussion

Bacterial growth and membrane protein expression which are regulated by iron (hemin) have been reported in several microorganisms).[7-11] Many of these proteins have been implicated as functional components of iron (hemin) uptake systems

in these species. Although the requirement for hemin has been known for many years,[12] little is known about the mechanism(s) by which *P. gingivalis* binds and uptakes hemin into the cell. There have also been no reports of the purification and characterization of an actual hemin-binding protein from *P. gingivalis*.

Several Gram negative bacteria are known to utilize hemin as a sole source of iron. Hemin-binding proteins have been identified in several of these species, including *Shigella flexnerii*,[13] *Bacteroides fragilis*,[10] *Neisseria gonorrheae*,[14] *Hemophilus influenzae*,[15–17] *Treponema denticola*,[7] and *P. gingivalis*.[5,18] However, only few of these proteins have been purified and characterized. In the study presented here, we were able to establish that a 30 kDa (unheated) cell envelope associated protein from *P. gingivalis* strain 381 bound hemin and was stained with TMBZ. The expression of this protein appeared to be tightly regulated by the level of hemin(iron) in the growth medium.

Functionally, Omp 26 of Bramanti and Holt[18] and Omp 32 of Smalley *et al.*[5] appear similar with respect to hemin. Bramanti and Holt[18] were unable to sequence Omp 26 because of N-terminal blockage, and Smalley *et al.*[5] did not provide any sequence data for their Omp 32. Internal amino acid sequence analysis of the CNBr digested fragment and a search of GenBank for proteins with similar internal amino acid sequence to the 24 kDa protein revealed no significant similarities, and we consider the 30 kDa (heated 24 kDa) membrane protein to represent a newly described hemin binding protein from *P. gingivalis* strain 381. To our knowledge, the study described here is the first to identify, purify and biochemically characterize a hemin-binding protein from *P. gingivalis*. Work is in progress to further characterize the molecular structure of this protein.

Materials and methods

Bacterial strain and culture conditions. *P. gingivalis* 381 was grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) Mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1 μg/ml menadione and 5 μg/ml hemin. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24 h, equivalent to late exponential growth phase. For hemin restriction (i.e. hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e. 7.7 μM hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. Iron limitation was achieved by the addition of 100 to 400 μM of the iron-chelating compound, 2,2-bipyridyl (BPD., Sigma Chemical Co., St. Louis, MO), into liquid growth medium containing 7.7 μM hemin. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. Culture purity was assessed by Gram staining and plating to solid medium.

Cell envelope preparation. Cells were harvested by centrifugation at 12,000 × ***g***, for 20 min at 4°C, washed three times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2 mM each of phenylmethylsulfonyl fluoride (PMSF), benzamidine and Na-P-tosyl-L-lysine chloromethyl ketone (TLCK). Cell envelopes were prepared by French pressure cell disruption of whole cells in PBS (pH 7.2) by four 15,000 lb/in^2 disruption cycles. The cell envelopes were removed after low-speed (10,000 × ***g***, 30 min) and high-speed (2,000,000 × ***g***, 2 h) centrifugation. Protein concentration was determined using the bicinchoninic acid (BCA) assay of (Pierce, Lockford, IL).

Polyacryamide gel electrophoresis. The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli[19] was employed for determination of protein distribution, and lithium dodecyl sulfate (LDS) PAGE was used

for the TMBZ staining studies. 12% acrylamide separating gels were routinely used. All gels were run with a 4% acrylamide stacking gel in a vertical slab gel apparatus (Hoefer Scientific, San Francisco, CA). Proteins were visualized by Coomassie Brilliant Blue-R-250 stain (CBB). The hemin-associated peroxidase activity of the cell envelope protein was determined by tetramethylbenzidine (TMBZ, Sigma Chemical Co., St. Louis, MO) staining of LDS-PAGE gels. The TMBZ staining was carried out as described by Stugard *et al.*[13]

Purification of hemin-binding protein. Isolation of hemin-binding protein from *P. gingivalis* 381 cell envelope was accomplished by solubilization in the zwitterionic detergent 3 [(3-chloramidopropyl)-dimethyl-ammonio] 1 propane sulfonate (CHAPS; Pierce, Rockford, IL). Cell envelopes from *P. gingivalis* 381 (passage 5) were isolated as described above, and solubilized by the addition of CHAPS to a final concentration of 1% (v/v) and incubated at 37°C, 1 h. The resulting suspension was ultracentrifuged at 100,000 ***g*** for 1h to pellet insoluble material, and the CHAPS-soluble fraction was either used immediately for the purification of hemin-binding protein or stored at −20°C until used. The hemin-binding protein referred to as 'unheated 30 kDa' (see Results) from *P. gingivalis* 381 was purified from the CHAPS-soluble membrane fraction of hemin-starved passage five cells by 1D-SDS-PAGE through a 12% gel employing a preparative comb with one reference well. The elution protocol of Hager and Burgess[20] was used. Purity of the isolated protein was confirmed by 1D SDS-PAGE.

Cyanogen bromide digestion and N-terminal sequencing. Initial experiments revealed that the unheated 30 kDa protein did not transfer well to a polyvinylidine difluoride (PVDF) membrane (Pro Blot, Applied Biosystems, Foster City, CA). Heating the 30 kDa protein at temperatures above 70°C resulted in the modification of the protein to a molecular weight of 24 kDa. In this heated, denatured condition the protein transferred quantitatively, and was used for N-terminal amino acid analysis. Attempts to directly sequence the N-terminus of the 24 kDa protein were unsuccessful because of a blocked N-terminus. Therefore, sequences were determined after cyanogen bromide (CNBr) digestion.

Cleavage by CNBr was carried out on the acetone precipitated 24 kDa protein. Several crystals of CNBr were added to 100 μl of 70% formic acid and swirled to dissolve. 50 μl of this solution was added to the acetone precipitated sample and allowed to proceed in the dark at room temperature, 16 h. The digest was dried under an N_2 stream and reduced to dryness in a SpeedVac SC 100 (Savant). The dried digest was dissolved in 1 × treatment buffer for SDS-PAGE, heated at 100°C for 5 min, electrophoresed through an exponential gradient gel (7.5 to 20%), and electroblotted to a PVDF membrane at 100 mA, 4 h. After transfer, the ProBlot membrane was removed from the transblotting sandwich and rinsed with deionized water. Protein bands on the ProBlot membrane was visualized by Amido black staining. The protein bands were excised from the dried membrane, and its N-terminal amino acid sequence was determined with an Applied Biosystems (Foster City, CA) Model 477A gas–liquid phase sequenator coupled to an on line high-performance liquid chromatography model 120A analyser.

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Porphyrin-Mediated Binding to Hemoglobin by the HA2 Domain of Cysteine Proteinases (Gingipains) and Hemagglutinins from the Periodontal Pathogen *Porphyromonas gingivalis*

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Heme binding and uptake are considered fundamental to the growth and virulence of the gram-negative periodontal pathogen *Porphyromonas gingivalis*. We therefore examined the potential role of the dominant *P. gingivalis* cysteine proteinases (gingipains) in the acquisition of heme from the environment. A recombinant hemoglobin-binding domain that is conserved between two predominant gingipains (domain HA2) demonstrated tight binding to hemin (K_d = 16 nM), and binding was inhibited by iron-free protoporphyrin IX (K_i = 2.5 μM). Hemoglobin binding to the gingipains and the recombinant HA2 (rHA2) domain (K_d = 2.1 nM) was also inhibited by protoporphyrin IX (K_i = 10 μM), demonstrating an essential interaction between the HA2 domain and the heme moiety in hemoglobin binding. Binding of rHA2 with either hemin, protoporphyrin IX, or hematoporphyrin was abolished by establishing covalent linkage of the protoporphyrin propionic acid side chains to fixed amines, demonstrating specific and directed binding of rHA2 to these protoporphyrins. A monoclonal antibody which recognizes a peptide epitope within the HA2 domain was employed to demonstrate that HA2-associated hemoglobin-binding activity was expressed and released by *P. gingivalis* cells in a batch culture, in parallel with proteinase activity. Cysteine proteinases from *P. gingivalis* appear to be multidomain proteins with functions for hemagglutination, erythrocyte lysis, proteolysis, and heme binding, as demonstrated here. Detailed understanding of the biochemical pathways for heme acquisition in *P. gingivalis* may allow precise targeting of this critical metabolic aspect for periodontal disease prevention.

Evidence for the potential importance of cysteine proteinases from *Porphyromonas gingivalis* in periodontal disease pathology is increasing. Periodontal disease affects the majority of adults to some degree and may be associated with significant systemic morbidity (2, 46), including dental infection and loss of teeth (36). *P. gingivalis* is implicated as an important periodontal pathogen by its high incidence and relative levels in human disease (1, 11) and by its virulence in monoinfected animals (14, 15). Virulence of *P. gingivalis* has been attributed to several components of the microorganism, including fimbriae (25, 37), short-chain volatile acids (12, 65), lipopolysaccharide (26, 58), collagenase activity (3, 39), and noncollagenolytic cysteine proteinase activity (8, 10, 54).

Cysteine proteinase activity may affect the remodeling of matrix proteins and disrupt the immune response by stimulating the collagen-degrading activity of host cells (8, 10, 62), degrading fibronectin (34), inactivating gamma interferon (68) and interleukins (6, 17), interfering with the complement cascade (63, 67), and degrading immunoglobulins (16, 52). Also, clotting and vascular permeability mechanisms may be disturbed (27, 28, 54), fibrinogen may be degraded (33, 54), and erythrocytes may be agglutinated and lysed (44, 56) by cysteine proteinase activity, possibly for the acquisition of metabolically necessary iron, heme, or porphyrin from hemoglobin. Numerous different *P. gingivalis* cysteine proteinases described in several reports have been demonstrated to be antigenically related (9, 47, 48) and the products of three related genes (41, 51). This unique family of enzymes, named gingipains, has two major gene products, Arg-gingipain-1 (RGP-1) and Lys-gingipain (KGP) (41), which prefer proteinacious substrates with an arginine or lysine in the P1 position, respectively.

Bacterial cysteine proteinase activity has been demonstrated within diseased periodontal pockets (13, 20), and epitopes of gingipains are detectable in clinical plaque samples from patients with adult periodontitis (unpublished data), so the gingipains are likely to be clinically relevant. The gingipains are expressed on the outer membrane of *P. gingivalis* and may also be released with vesicles or as soluble proteins (9, 18, 24). Gingipains have been suggested to account for up to 85% of trypsin-like proteolytic activity in a *P. gingivalis* culture (49), and under certain growth conditions in vitro, these enzymes can accumulate to become the most abundant *P. gingivalis* proteins in a culture (9).

The catalytic domains of RGP-1 and KGP constitute approximately one-third of the translated protein products. The remaining two-thirds of these two gingipain molecules consist of four COOH-terminal domains (HA1 to HA4) which are highly homologous between these two predominant gingipains (Fig. 1). These noncatalytic COOH-terminal domains were originally named hemagglutinin (HA) domains because at least one was thought to participate in hemagglutination (47). They may each be separated posttranslationally from the catalytic domain and from one another, presumably through au-

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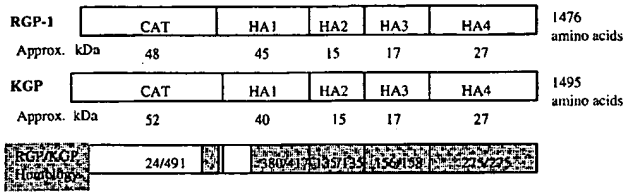

FIG. 1. Domain structure and homologies between the gingipains RGP-1 and KGP. CAT represents the putative catalytic domain. Shaded areas represent regions of >98% amino acid identity between the two gingipains. Each fraction represents the degree of identity for each RGP-1 domain. Approx. kDa, approximate molecular mass in kilodaltons.

tolysis some time after logarithmic growth in vitro (9, 59). The functions of the first, third, and fourth HA domains are unknown. The second HA domain (HA2) has recently been implicated in hemoglobin binding (19, 43). Because all of the domains of the gingipains are found together predominately in loose, noncovalent associations with one another after hydrolytic separation (9, 59), the gingipains appear to be multifunctional proteins for aggregation of erythrocytes and then lysing of these cells to obtain hemoglobin for the acquisition of iron, heme, or porphyrin.

P. gingivalis (formerly *Bacteroides* sp.) can utilize inorganic iron, free or protein-associated heme, or organic iron sources such as transferrin (5). Several investigators have previously shown that *P. gingivalis* binds to and internalizes hemin with various affinities and at various rates (4, 21, 53, 57, 60, 64). These earlier reports suggest that there are at least two heme-binding proteins of *P. gingivalis* with different affinities for hemin which may respond to environmental changes by rapidly changing their position or associations within the outer membrane.

Hemin binding and uptake appear to be related to the regulation of proteinase and fimbriae expression and to vesicle formation (7, 38, 40) and were recently proposed to establish an antioxidative shield for protection from oxidative radicals (61). Binding of protoporphyrin IX in *P. gingivalis* was also implied by competition with labelled hemin (4, 64), and protoporphyrin IX was reported to support growth (53). Protoporphyrin IX limitation was shown to be coordinated with phenotypic expression of proteinase activity (42). Hemin binding by *P. gingivalis* may therefore represent a capacity for protoporphyrin binding.

Recently, Nakayama et al. have isolated a hemoglobin-binding protein associated with the outer membrane of *P. gingivalis* and identified this protein as one homologous with the HA2 domain of the gingipains (43). In that report, adsorption of hemoglobin to whole *P. gingivalis* cells was associated with the presence of the HA2 domain. Also, hemin accumulation within the *P. gingivalis* cells was shown to be dependent on functional expression of KGP (45). The HA2 gingipain domain may therefore function as a hemoglobin-binding domain in *P. gingivalis*.

Understanding the molecular and biochemical mechanisms involved in key regulatory pathways is paramount in developing strategies for control of disease. In this study, we obtained evidence, by using a monoclonal antibody (MAb) which recognizes the hemoglobin-binding (HA2) domain of *P. gingivalis* cysteine proteinases, that the HA2 domain can bind to hemoglobin primarily and specifically through a portion of the heme moiety that is surface exposed in the hemoglobin structure. We also found that the unique epitope of MAb 5A1 within this heme-binding domain was expressed in parallel with hemoglobin-binding activity and proteinase activity in cellular and cell-free culture fractions of *P. gingivalis*.

MATERIALS AND METHODS

RGP-1 and KGP isolation. Polydomain RGP-1 and KGP were isolated and characterized as previously described (68) by arginine-Sepharose affinity chromatography of detergent-extracted *P. gingivalis* ATCC 33277 cells. Alternatively, polydomain RGP-1 and KGP were isolated as previously described (9) by arginine-Sepharose affinity chromatography from cell-free supernatant of a 10-day *P. gingivalis* batch culture.

Enzyme activity assays. The proteinase activities of *P. gingivalis* culture fractions were measured by using the substrates *N-tert*-butoxycarbonyl-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin or *N-tert*-butoxycarbonyl-Glu-Lys-Lys-7-amido-4-methylcoumarin at 30°C in Tris buffer without added reducing agents. Substrate hydrolysis was monitored over time by measuring A_{460} with a 380-nm excitation beam on a Perkin-Elmer LS 50B luminescence spectrophotometer.

Development of MAbs 5A1 and 1IB2. Antigingipain MAbs 5A1 and 1IB2 were prepared in mice as previously described (9).

Expression and purification of recombinant HA2 (rHA2). Forward and reverse primers (AACCTGCAGCGCGCAGACTTCACGG and GGAAGCCAA TGGCGCCAAAAGATCTAGT) were designed to amplify the HA2 domain from the *P. gingivalis* RGP-1 proteinase gene (accession no. U15282). Restriction sites for *Pst*I and *Bgl*II were designed into the 5′ ends of the primers to facilitate cloning. The digested PCR product was ligated into the QIAexpressionist type III construct providing a six-His tag at the COOH terminus (Qiagen Corp.). Transformation of the ligated construct was performed by electroporation into *Escherichia coli* NM522 cells. *E. coli* cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. Cells were harvested and resuspended to 5 ml/g (wet weight) in buffer A (8 M urea, 0.1 mM NaH_2PO_4, 0.01 mM Tris-HCl, pH 7.9). The cells were stirred for 2 h at room temperature, taking care to avoid foaming. This cell lysate was subjected to centrifugation at 31,000 × *g* for 30 min at room temperature to pellet the cellular debris, and then the supernatant was subjected to ultracentrifugation at 130,000 × *g* for 2 h. The clarified lysate was loaded onto a nickel-nitrilotriacetic acid column (Qiagen Corp.), pre-equilibrated with buffer A. The nickel-nitrilotriacetic acid column was washed with buffer A until the baseline was reached. The protein was refolded on this column by running a linear gradient of urea from 8 to 0 M in 20 mM Tris-HCl–500 mM NaCl–10% glycerol (pH 7.9). The protein was then eluted with 50 mM Tris-HCl–500 mM NaCl–10% glycerol–250 mM imidazole (pH 7.9). The eluant was diluted 100-fold in 50 mM sodium acetate buffer (pH 5.5) and applied to a hemoglobin-agarose column pre-equilibrated with the dilution buffer. After loading, the column was washed with the same buffer until the baseline was reached and then the hemoglobin-binding protein was eluted with 50 mM Tris-HCl (pH 9). Protein concentrations were determined by Coomassie dye binding using bovine serum albumin as the standard.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using 12% gels with 4% stackers by the method of Laemmli (32). All samples were diluted with SDS sample buffer before electrophoresis with (reducing) or without 2-mercaptoethanol. Western blotting was performed by the method of Towbin et al. (66), and proteins were transferred from the gels to polyvinylidene difluoride (PVDF) paper (Bio-Rad) with 300 mA for 1 h. Blots were blocked with 0.1% bovine serum albumin in 20 mM Tris-HCl with 500 mM NaCl containing 0.1% Tween 20 (TBS/Tween). An alkaline phosphatase (AP) conjugate of rabbit anti-mouse immunoglobulin G (Dako Corp.) was used as a secondary antibody. Blots were washed with TBS/Tween between antibody applications. The substrate for AP was nitroblue tetrazolium in excess with 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), and color was developed in 5 mM Tris (pH 9.5).

NH_2-terminal amino acid sequencing of proteins resolved by SDS-PAGE was performed as previously described (9).

ELISA. Enzyme-linked immunosorbant assays (ELISA) were performed in polystyrene microtiter wells. Proteins were used to coat the surfaces in 2.7 mM KCl–1.5 mM KH_2PO_4–137 mM NaCl–8.1 mM Na_2HPO_4 (PBS) with 10 mM sodium azide (PBS/N_3). All wells were blocked and washed in PBS with 0.1% Tween 20 (PBS/Tween). Primary murine antibodies were applied in PBS/Tween at a concentration of 0.5 μg/ml for at least 1 h. Secondary goat anti-mouse antibodies conjugated with AP (Dako Corp.) were applied at a concentration of 1.1 μg/ml for 30 min, and then AP activity was monitored at 414 nm by hydrolysis of the substrate 4-nitrophenylphosphate (Boehringer GmbH, Mannheim, Germany) in 5 mM Tris (pH 9.5) by using a Titertek Twinreader PLUS photometer (absorbance maximum of 3.0 ELISA units). Mean apparent dissociation constants (K_ds) were derived by solid-phase ELISA as previously described (50) and are accompanied by standard errors of the means.

Ligand-binding assay. The ligand-binding assay was a variant of the ELISA in which the ligand (i.e., hemin or hemoglobin) that had been used to coat the wells in PBS/N_3 was subsequently allowed to bind to a second ligand-binding protein (i.e., rHA2 or gingipains) in PBS/Tween. The ligand-binding protein was then detected with MAb 5A1 or 1IB2, followed by a rabbit anti-mouse AP conjugate, and developed as already described for ELISA. Bovine hemoglobin was used in these experiments. Hemin was from stock solutions dissolved in 0.1 N NaOH,

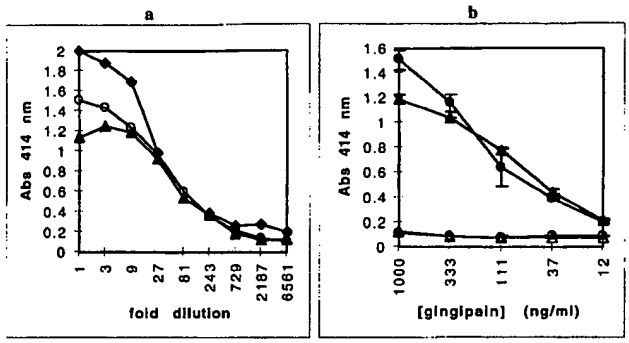

FIG. 2. Hemoglobin binding by rHA2, RGP-1, and KGP. (a) Microtiter wells were coated with hemoglobin and then incubated with threefold dilutions of purified rHA2 at 2.5 μg/ml (◆), RGP-1 at 5 μg/ml (○), or KGP at 5 μg/ml (▲). Association of rHA2 with hemoglobin was measured with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. (b) Hemoglobin binding by native, but not denatured, gingipains. Wells were coated with hemoglobin and then incubated overnight with dilutions of either RGP-1 (●), KGP (▲), RGP-1 denatured by boiling (○), or KGP denatured by boiling (△). For this experiment, native or denatured gingipains that bound to hemoglobin were recognized by MAb IIB2, which specifically detects both native and denatured gingipains. Primary antibody IIB2 was followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of three separate experiments. Abs, absorbance.

and although the NaOH would replace the chloride ion of hemin with a hydroxylate ion (hematin), the term hemin will be used for this compound throughout this report. The K_d and apparent inhibition constant (K_i) for ligand binding were derived as previously described (50) in these assays by using serial dilutions of the ligand-binding protein or competitor, respectively, with even amounts of coated ligand. The reported results are means accompanied by the standard errors of the means.

Peptide synthesis. Peptides were synthesized by Chiron Mimotopes with terminal amines and carboxylic acids. The peptide 1 sequence was ALNPDNYLISKDVTG, and the peptide 2 sequence was GEAPAEWTTIDADGDGQGWL.

Materials. All chemicals and compounds were purchased from Sigma unless otherwise specified.

Statistics. Statistical differences between measurements of the gingipains and rHA2 were determined with one-tailed Student t tests.

RESULTS

The polydomain RGP-1 and KGP isolated from 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-extracted *P. gingivalis* cells possessed SDS-PAGE profiles, NH_2-terminal sequences, proteolytic activities, and inhibition profiles characteristic of gingipain-like molecules previously described by us (9, 68) and others (3, 47, 54) (data not shown).

The HA2 domain was cloned, expressed, and purified as a six-His tag fusion. Nucleic acid and NH_2-terminal amino acid sequencing verified the identities of the clone and the expressed protein, respectively, as the HA2 domain of RGP-1 (data not shown).

Hemoglobin is bound by rHA2 and by native but not denatured RGP-1 and KGP. In the solid-phase ligand-binding assay, rHA2, RGP-1, and KGP each bound to hemoglobin (Fig. 2a). As MAb 5A1 was used to detect rHA2 bound to hemoglobin and did not interfere with this binding, it was evident that the epitope for MAb 5A1 within the HA2 domain was separate from the hemoglobin-binding site of HA2. The hemoglobin-binding affinities of rHA2, RGP-1, and KGP (K_d = 2.1 ± 0.6 nM) were similar (P = 0.24), and the binding curves of neither rHA2 nor the gingipains were indicative of multisite binding (Fig. 2a). High-affinity binding to hemoglobin at a single site within only the HA2 domain of both native RGP-1 and KGP is sufficient to account for these observations. The binding site for hemoglobin within the gingipains appeared to be associated with a higher-order protein structure, since denaturation of RGP-1 and KGP by boiling effectively eliminated their ability to bind hemoglobin (Fig. 2b).

Hemoglobin binding of the HA2 domain is mediated through the heme moiety. To begin characterizing the binding between rHA2 and hemoglobin, we examined the binding between rHA2 and hemin, as well as binding to hemoglobin degraded by proteinase K. rHA2 bound not only to wells coated with hemoglobin but also to wells coated with hemin or with proteolytically degraded hemoglobin (Fig. 3a). Binding of the rHA2 domain to hemin-coated wells was approximately eightfold weaker than binding to hemoglobin in solid-phase assays (K_d = 16 ± 1 nM) (Fig. 3b).

The HA2 domain binds the porphyrin ring structure. To dissect the binding of the rHA2 domain to hemin, the K_is of iron-free protoporphyrin IX in solution phase competition assays were determined. By using the standard ligand-binding assay described herein, rHA2 or the gingipains were preincubated with dilutions of protoporphyrin IX and then allowed to bind to the hemin-coated wells. Binding of the gingipains or rHA2 to hemin was inhibited by the addition of protoporphyrin IX (K_i = 2.5 ± 0.3 μM) (Fig. 4a). The apparent K_i values of rHA2 and the gingipains were similar (P = 0.42). These data indicated that binding of rHA2 or the gingipains to hemin was specific for some aspect of the protoporphyrin ring. Importantly, binding of rHA2 or the gingipains to hemoglobin was also inhibited by protoporphyrin IX (Fig. 4b) (K_i = 10 ± 2 μM) and preincubation with the protoporphyrin effectively eliminated binding to hemoglobin.

Directed protoporphyrin binding by rHA2. Examination of the hemoglobin crystal structure indicated that only the region of the heme moiety possessing the propionate functional groups (Fig. 5) would be exposed for possible protein-protein contact. We therefore reasoned that blocking access to the acidic region of protoporphyrin molecules would have an effect on rHA2 binding and allow more specific characterization of binding between the HA2 domain and the porphyrin ring. In a modification of the ligand-binding assay system described

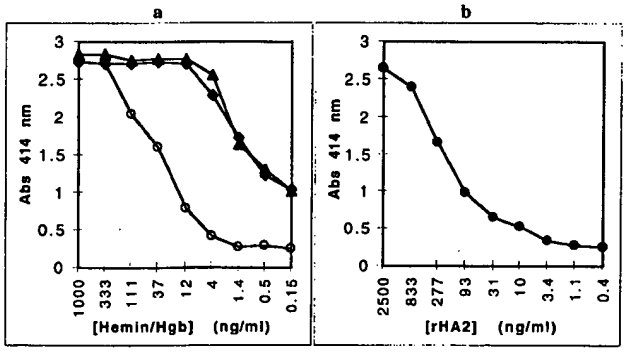

FIG. 3. Binding of the HA2 domain to the heme moiety. (a) Binding of rHA2 to dilutions of hemin (◆), hemoglobin (Hgb) (○), or hemoglobin degraded by proteinase K (▲). Microtiter wells were coated with dilutions of samples, and then overnight binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. The absence of contaminating protein within 90 μg of the hemin preparation and the absence of nondegraded subunits of hemoglobin remaining after proteinase K treatment were verified by SDS-PAGE (data not shown). (b) Binding of rHA2 to hemin. Microtiter wells were coated with hemin, and overnight binding of rHA2 dilutions was detected with MAb 5A1 as described above. These data are representative of two separate experiments. Abs, absorbance.

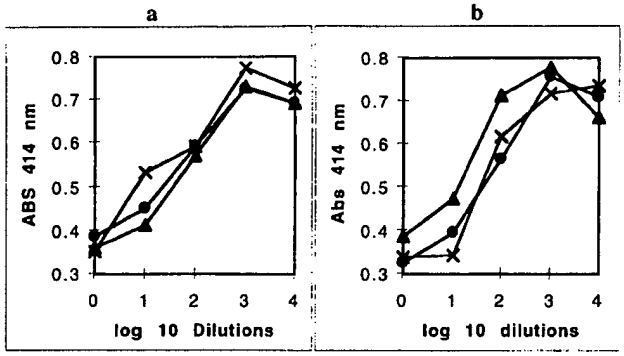

FIG. 4. Inhibition of hemin or hemoglobin binding. Microtiter wells were coated overnight with hemin (a) or hemoglobin (b). rHA2 in *E. coli* lysate (100-fold dilution) (×), RGP-1 at 65 ng/ml (●) or KGP at 65 ng/ml (▲) was preincubated with dilutions of 300 μM protoporphyrin IX for 1 h and then transferred to the ligand-coated plates for overnight incubation. Binding of rHA2 or the gingipains to coated wells was detected with MAb 5A1 or 1IB2, respectively, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of two separate experiments. The absence of contaminating protein in a 90-μg protoporphyrin IX preparation was verified by SDS-PAGE and Coomassie dye binding (data not shown). ABS, absorbance.

above, surfaces were first coated with ethylene diamine to provide fixed, free, primary amines for carbodiimide linkage of carboxylic acid groups. Hemin, protoporphyrin IX, and hematoporphyrin bound to wells coated with ethylene diamine with or without carbodiimide treatment, as determined by A_{414} measurement (Fig. 5, striped bars). rHA2 binding to the carbodiimide-treated porphyrins in the wells was almost eliminated, however, compared to the relatively greater association of rHA2 with the nonderivatized porphyrins (Fig. 5, solid bars). These data indicated that the rHA2 domain specifically recognized the three porphyrin compounds in the region of the propionic acid groups, as we were able to block rHA2 binding by directionally attaching the carboxylic acids of hemin, protoporphyrin IX, or hematoporphyrin to fixed amines. Since the heme moiety within hemoglobin is almost identical to these porphyrin molecules, the data suggested that the heme moiety of hemoglobin was bound by rHA2 and by the HA2 domain of the gingipains in a similar, directed, high-affinity manner.

The MAb 5A1 epitope is recognized in the rHA2 domain and in denatured, but not native, RGP-1 and KGP. In ELISA, MAb 5A1 bound to rHA2 with high affinity (K_d = 2.2 × 10^{-10} ± 0.5 × 10^{-10} M) (Fig. 6a). MAb 5A1 also bound to denatured RGP-1 and KGP but did not bind to the native gingipains isolated from CHAPS-extracted *P. gingivalis* cells (Fig. 6b). Soluble high-molecular-weight aggregates of gingipain domains isolated from the cell-free fraction of a *P. gingivalis* batch culture by arginine-Sepharose affinity chromatography (9) were, however, recognized by MAb 5A1 (K_d = 1.7 × 10^{-10} ± 0.6 × 10^{-10} M) (Fig. 6c). The similarity of the dissociation constants (P = 0.36) and binding curves suggested that MAb 5A1 recognized the same HA2 epitope in these polydomain gingipains as in rHA2.

The MAb 5A1 epitope is represented by an amino acid sequence within the HA2 gingipain domain. By use of linear synthetic peptides, the epitope of MAb 5A1 was determined to be associated with the peptide ALNPDNYLISKDVTG (K_d = 3.8 nM), which represents amino acids 1215 to 1229 of translated KGP within the HA2 domain (Fig. 7, peptide 1). Dot blot analysis on a PVDF membrane confirmed the unique immunoreactivity of this peptide with MAb 5A1 (data not shown). A search of the SwissProt database for the linear sequence of peptide 1 or of the GenBank database by using the deduced nucleic acid sequence of this epitope resulted in no molecules with perfect homology to the peptide other than the gingipains and HagA, a large HA with regions of identity to the entire HA2 domain.

Correlation of HA2 domain immunoreactivity with hemoglobin binding in a *P. gingivalis* culture. Detection of the HA2 epitope with MAb 5A1 in unfractionated *P. gingivalis* samples was correlated with hemoglobin binding. Because proteinase activity and gingipain expression have been shown to progressively change during the course of an extended *P. gingivalis* batch culture (9), we examined cell-associated and extracellular fractions during 8 days of culture. Both Arg- and Lys-

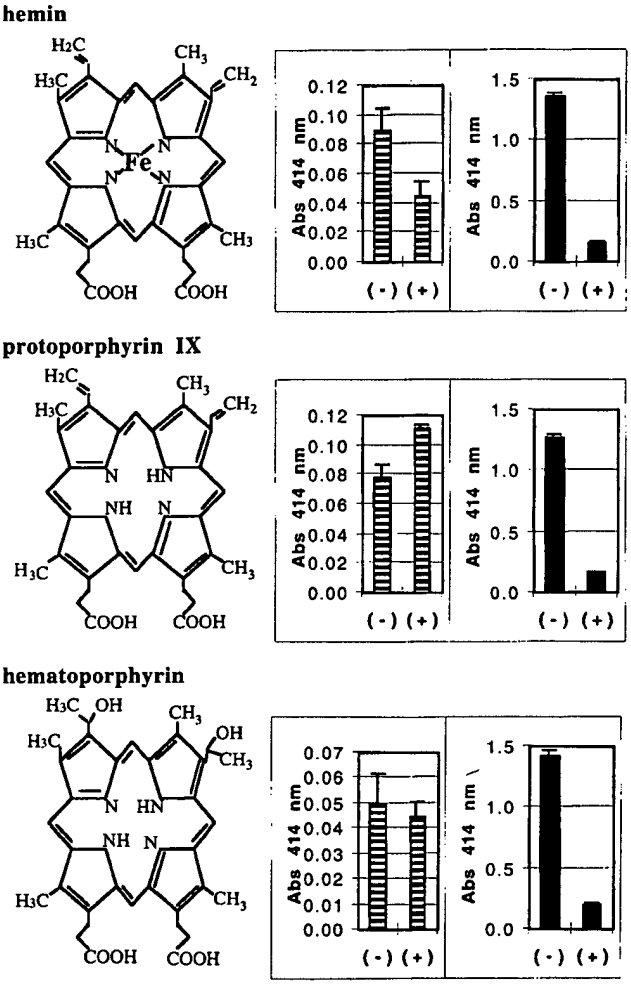

FIG. 5. Directed porphyrin binding by rHA2. Microtiter wells were coated with 100 mM ethylene diamine (pH 4.7) and then incubated with hemin, protoporphyrin IX, or hematoporphyrin at 90 μg/ml overnight in 50% dimethyl formamide in the presence (+) or absence (−) of 10 mM carbodiimide. Wells were washed four times with water, and then the amount of porphyrin bound to the wells was determined by measuring absorbance (Abs) at 414 nm (striped bars). Wells were blocked with PBS/Tween and then incubated with rHA2 at 125 ng/ml overnight. Binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody (solid bars). Error bars represent standard deviations of absorbance measurements. Diagrams of the chemical structures of hemin, protoporphyrin IX, and hematoporphyrin are presented adjacent to the corresponding data.

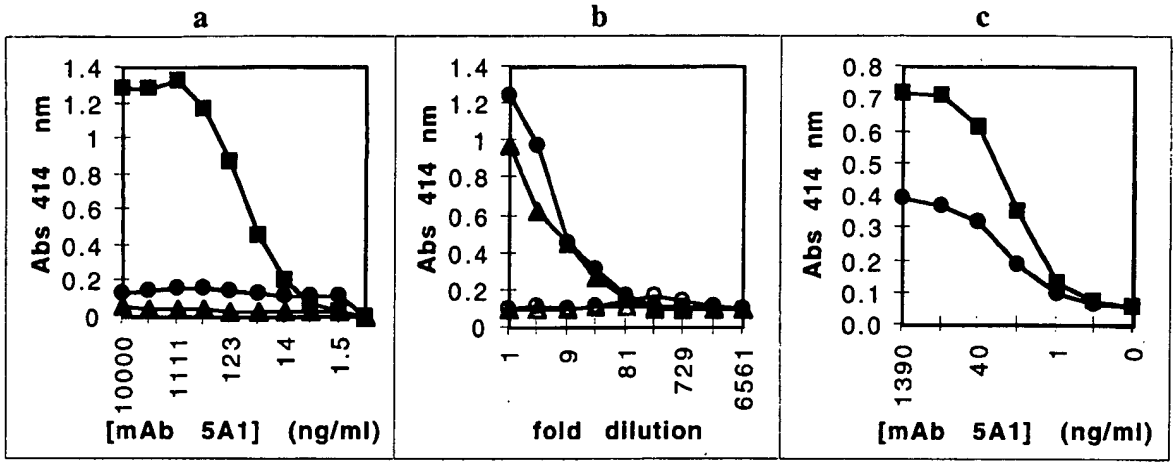

FIG. 6. Measurement of high-affinity binding of MAb 5A1 with rHA2, denatured but not native gingipains, and gingipains from the culture supernatant. (a) RGP-1 (●), KGP (▲), or rHA2 in crude *E. coli* lysate (■) was used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. (b) Dilutions of RGP-1 (○), KGP (△), or heat-denatured RGP-1 (●) or KGP (▲) were used to coat microtiter wells with threefold dilutions from 10 μg/ml and then incubated with MAb 5A1. (c) Purified rHA2 (■) or purified high-molecular-weight aggregates of gingipain domains isolated from culture supernatant (●) were used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. These data are representative of three separate experiments. Abs, absorbance.

specific proteinase activities of the *P. gingivalis* cells peaked near day 3 of culture (Fig. 8a and b, triangles). Proteinase activities of the cell-free culture supernatants steadily rose throughout the culture period (Fig. 8a and b, squares).

Immunoreactive protein in the cell-free conditioned culture medium detected with MAb 5A1 steadily accumulated throughout the 8-day culture period, similar to proteolytic activity (Fig. 8c, open squares). Immunoreactive protein associated with hemoglobin binding in this supernatant fraction also increased steadily throughout the extended culture in a parallel manner (Fig. 8c, closed squares). In the cellular fraction of the *P. gingivalis* culture, expression of immunoreactive protein increased early during the culture period with a peak near day 3 followed by a slight decrease and then an increase to peak levels again by day 7, similar to the proteolytic activity of this fraction (Fig. 8c, open triangles). Immunoreactive protein associated with hemoglobin binding in the cellular fraction followed a parallel pattern of expression (Fig. 8c, closed triangles). These data demonstrated that detection of protein immunoreactive with MAb 5A1 in crude cellular and extracellular fractions of a *P. gingivalis* culture was directly associated with hemoglobin binding, suggesting that MAb 5A1 specifically recognized the hemoglobin-binding HA2 domain within the *P. gingivalis* culture. Also, the data demonstrated a profile of HA2 domain expression and hemoglobin-binding activity similar to the profile of cellular and extracellular proteolytic activity expressed by *P. gingivalis*.

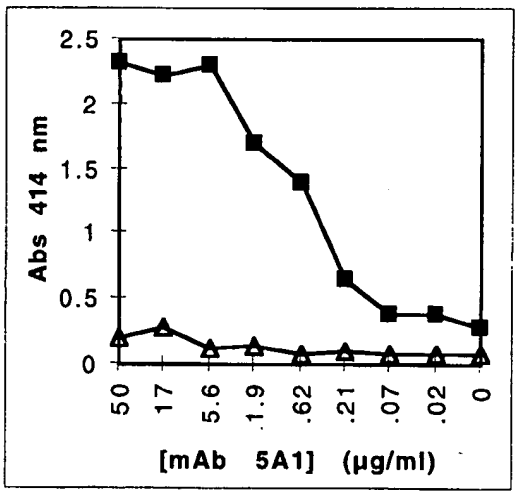

FIG. 7. Immunoreactivity of synthetic peptides with MAb 5A1. ELISA demonstrating selective immunoreactivity of MAb 5A1 with peptide 1. Peptide 1 (■) or 2 (△) was used to coat microtiter plates at a concentration of 5 μg/ml, incubated overnight, and then incubated with dilutions of MAb 5A1. These data are representative of two separate experiments. Abs, absorbance.

DISCUSSION

Control of *P. gingivalis* growth to prevent periodontal pathology might be achieved by interference with one or more pathways for obtaining heme. To this end, we have reported on a MAb which recognizes an epitope within the hemoglobin-binding domain of the abundant *P. gingivalis* cysteine proteinases, named gingipains, and demonstrated increasing levels of this HA2 domain associated with hemoglobin binding and proteinase activity in an extended *P. gingivalis* culture. Further, we have characterized the binding between the HA2 domain and hemoglobin, suggesting that binding is mediated in large part by specific recognition of the porphyrin ring of the heme moiety within hemoglobin.

The hemoglobin-binding affinities of RGP-1, KGP, and the HA2 domain measured in our experiments were similar. Also, binding curves for these interactions were typical of single-site binding, which is consistent with the idea that the HA2 domain of the cell-derived gingipains is solely responsible for hemoglobin binding. The similarity of the inhibition profiles for the gingipains to that of rHA2 further suggested that mediation of gingipain binding to heme was through only the HA2 domain. These data do not, however, rule out other possible heme-binding sites in the gingipains with affinity identical to that of HA2.

Hemoglobin binding by the separated catalytic domain of KGP was recently demonstrated (31). Our data, obtained by using polydomain gingipains, did not provide evidence for this

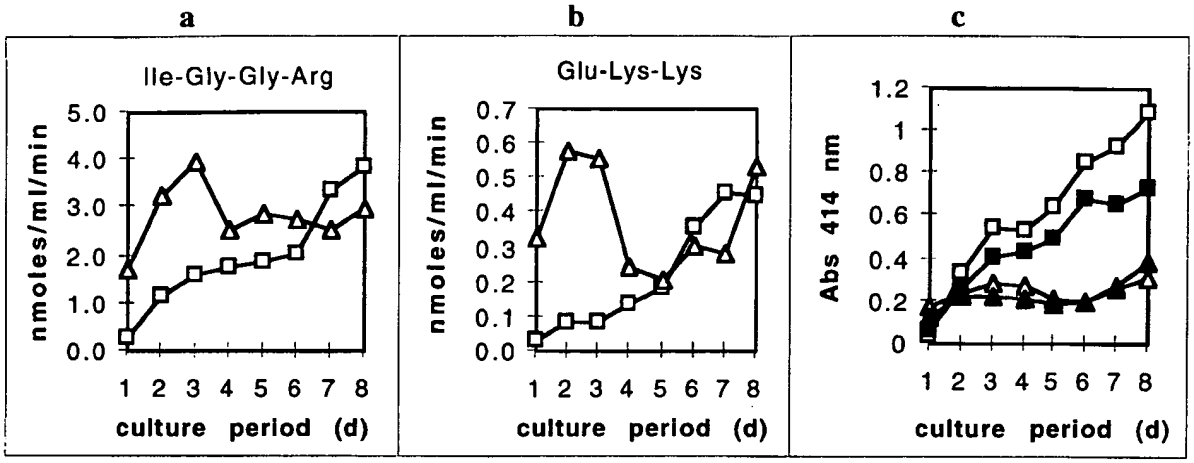

FIG. 8. Expression of HA2-related immunoreactive hemoglobin-binding protein from *P. gingivalis*. Aliquots of *P. gingivalis* culture medium were removed daily during a period of 8 days (d), immediately separated into a cell pellet and culture supernatant, and then frozen until use. The OD_{660} and purity of the culture were measured daily. The cell pellets were dispersed evenly into 1 ml of PBS/N_3. (a and b) Arg- and Lys-specific proteinase activities, respectively, of the cell-free culture supernatant (□) and cellular fraction (△) were measured as described in Materials and Methods. Measurements of the cellular fractions were normalized to the culture densities (OD_{660}) recorded daily. (c) The HA2 domain (1/243 dilution; □) and the HA2 domain associated with hemoglobin binding (1/81 dilution; ■) in culture supernatants were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. In *P. gingivalis* whole-cell fractions, the HA2 domain (1/243 dilution; △) and the HA2 domain associated with hemoglobin binding (1/9 dilution; ▲) were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. Measurements of the cell-associated fractions were normalized to the culture densities (OD_{660}) recorded daily. The corresponding background immunoreactivity with murine anti-human CD-19 immunoglobulin G was subtracted from each measurement. These data are representative of two separate experiments in which the patterns of expression were similar. Abs, absorbance.

second hemoglobin-binding site. It is likely, however, that separated domains of the gingipains behave differently than when associated either noncovalently or within a single polydomain polypeptide. The inability of MAbs which recognize either isolated gingipain domains or peptides to recognize the larger polydomain gingipains of cells exemplifies this potential (this report and reference 22).

Apparent dissociation constants in the nanomolar range represented significantly tighter binding of the HA2 domain to hemoglobin than previously reported (43). Further, this relatively tight binding in our experiments was measured at a nearly neutral pH and not at the pH maximum for binding of 5.5 reported earlier. Differences in experimental systems for measuring binding may account for this discrepancy.

Protoporphyrin IX inhibited binding of rHA2 to hemin. Also, protoporphyrin IX and hemin did not differ statistically in the ability to inhibit the binding of rHA2 to hemin (data not shown). This indicated that the sequestering of porphyrin by HA2 functioned independently of iron. The side chain groups of the porphyrin also did not appear to determine HA2 binding. Hematoporphyrin differs from protoporphyrin IX only by the hydroxylation of the two side chain ethylene groups. These groups are located opposite the positions of propionate groups across the plane of the porphyrin. As the binding to HA2 of these two porphyrins was comparably strong (Fig. 5), it can be concluded that HA2 binding was insensitive to the nature of the chemical groups attached at these positions. This contrasts with the blocking of rHA2 binding in both hematoporphyrin and protoporphyrin IX by directional attachment through chemical modification of the propionate groups.

The iron chelator 2,2′-dipyridal at a concentration of 2 mM also inhibited the binding of rHA2 to hemin, although the K_i of the dipyridal was 200-fold higher than the K_i of protoporphyrin IX (data not shown). This may indicate that rHA2 also had some weak interaction with the iron, but direct steric interference by the dipyridal in the absence of direct iron binding by rHA2 could also be considered.

Binding of hemin by the rHA2 domain was eightfold weaker than that of hemoglobin, although it would be expected to be similar if binding of the HA2 domain to hemoglobin occurred solely through the porphyrin ring of the heme ligand. Competition experiments demonstrated that protoporphyrin IX also inhibited hemoglobin binding, although it was approximately fourfold less competitive than in hemin-binding assays. A portion of the hemoglobin polypeptide may, therefore, contribute to the interaction of HA2 with hemoglobin in a cooperative manner. Because protoporphyrin IX alone completely blocked the interactions between rHA2 or the gingipains and hemoglobin, however, binding between the HA2 domain and the heme moiety must have been essential for the maintenance of this cooperative hemoglobin binding. Alternatively, the weaker binding of rHA2 with hemin in these experiments might also be due to the possibility that iron-protoporphyrins in solution can dimerize, ruffle, or associate differently than when bound to hemoglobin (23, 29, 55). Further, the HA2-binding region of the relatively smaller hemin ligand when bound directly to a surface may be less sterically accessible to the HA2 domain than when heme is presented and supported as part of a large globular protein where the propionate groups and the adjoining rim of the porphyrin ring protrude slightly beyond the surface of the protein (35).

Gingipains recovered from the culture supernatant subsequent to the first day of growth were previously shown to consist of noncovalently aggregated lower-molecular-weight domain fragments of the gingipains (9, 59). Although MAb 5A1 did not recognize native gingipains purified from solubilized *P. gingivalis* cells, MAb 5A1 did detect gingipain domain aggregates purified from the culture supernatant. This is not surprising, considering that the antibodies were made against the domain fragments of these gingipains (9), and it demonstrates potential differences between high-molecular-weight gingipains recovered by various means.

It is not known whether the HA2 domain was recognized in our cultures as a separate domain, as implicated by the isola-

tion of the separate HA2 domain from envelope fractions (19), or whether the HA2 domain was part of a polydomain complex of gingipain fragments or derived from the *hagA* gene product. Since the gingipains would be required for hydrolytic release of the HA2 domain from the *hagA* gene product, as well as from the gingipains themselves (43, 45), analysis of porphyrin binding in *hagA* knockout strains of *P. gingivalis* is needed to address this question. Our data demonstrated that the presence of the HA2 domain released by the cells paralleled proteinase activity, as well as hemoglobin-binding activity, suggesting that the hemoglobin-binding HA2 domain was derived from the gingipains. Although these data do not directly implicate the HA2 domain in iron, heme, or porphyrin acquisition by the *P. gingivalis* organism, the HA2 domain was associated with hemoglobin binding and could be considered a specific target for interference with heme acquisition by *P. gingivalis*. An HA2-specific antibody which blocks HA2 binding to heme or hemoglobin might be useful in dissecting the role of this porphyrin-binding domain in whole-cell metabolism and virulence.

Hemagglutination was the original function ascribed to the four COOH-terminal domains of the gingipains (47). Although the HA2 domain functions as a porphyrin-binding domain, it might, in addition, participate in hemagglutination. The separate rHA2 domain, at a concentration of 2 μg/ml, did not agglutinate erythrocytes, however, and MAb 5A1, which bound to the HA2 domain, did not inhibit the hemagglutination capacity of whole *P. gingivalis* cells (data not shown). We are currently investigating the functions of each gingipain HA domain.

Sequence analysis and trypsin susceptibility make the hemin-binding Omp26 described by Bramanti and Holt clearly different from the HA2 domain (4, 30). We have therefore identified a second hemin-binding protein in *P. gingivalis*. Interestingly, a recent independent analysis of hemin binding by whole cells of *P. gingivalis* described two different affinities (64). Now we have demonstrated that hemin- or hemoglobin-binding activity is also released by *P. gingivalis* in batch cultures. It is not immediately clear what advantage *P. gingivalis* would gain by releasing heme-binding activity, but it may be speculated, considering the recovery of the separate HA2 domain from the outer membrane (43), that soluble HA2 might reassociate with other gingipain domains on the *P. gingivalis* cells after scavenging and binding to heme or hemoglobin. A specific association of the HA2 domain with an active catalytic domain may be required for removal of the heme moiety from hemoglobin.

Characterization of the binding between the rHA2 domain and porphyrins should allow design of efficient affinity ligands for purification of HA2 and allow structure-based design of inhibitors of heme or hemoglobin binding. Heme acquisition is considered to be fundamental to the growth of *P. gingivalis*, and intervention with specific agents to disrupt pathways for heme binding or uptake may allow the eventual control or prevention of periodontal disease.

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Short communication

Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*

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Kim, S.-J. (Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284, U.S.A.), L. Chu and S. C. Holt. Isolation and characterization of a hemin-binding cell envelope protein from *Porphyromonas gingivalis*. *Microbial Pathogenesis* 1996; **21**, 65–70.

A 30 kDa (heated 24 kDa) hemin-binding protein whose expression is both hemin and iron regulated was identified and purified in *Porphyromonas gingivalis* 381. A strong hemin-binding function was found by LDS-PAGE and TMBZ staining when cells were grown under hemin (iron)-limited conditions. N-terminal amino acid sequence analysis of CNBr-digested 24 kDa hemin binding protein revealed that this protein belongs to a new, so far undescribed hemin-binding class of proteins.

Key words: Hemin; *Porphyromonas gingivalis*; TMBZ; outer membranes.

Introduction

While there are several studies which have dealt with the expression of hemin-regulated proteins in the oral pathogen, *Porphyromonas gingivalis*,[1–5] there are few studies which have demonstrated actual hemin-binding. Smalley *et al.*[5] identified a major tetramethylbenzidine(TMBZ) staining 32 kDa (unheated) protein in hemin-limited *P. gingivalis* W50, while Grenier[6] demonstrated that the hemin-binding property of *P. gingivalis* ATCC 33277 is mediated by the lipopolysaccharides, particularly the Lipid A region. In the study presented here, we have identified, purified, and characterized a putative hemin binding protein in *P. gingivalis* 381.

Results

Identification of hemin-binding protein

In Fig. 1(a), the upregulation of the 56 and 30 kDa proteins (unheated) is very clear. TMBZ staining [Fig. 1(b)] revealed the only protein to bind the stain was at 30 kDa when cells were grown under hemin(iron) restriction. Note in Fig. 1(b) that

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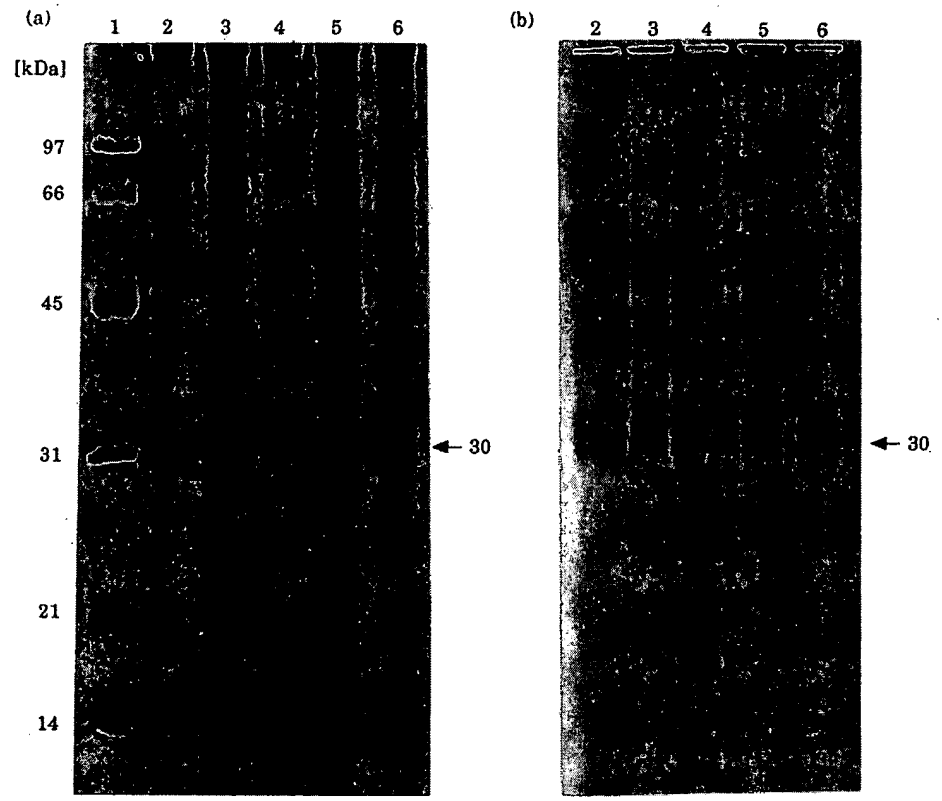

Fig. 1. Coomassie Brilliant blue stained (a) and tetramethylbenzidine (TMBZ) stained (b) LDS-PAGE of cell envelopes from *P. gingivalis* 381. Protein (70 μg) was applied to each lane following incubation with hemin. Lane 1, low mol.wt. standards; lane 2, cells grown + 7.7 μM hemin; lane 3 passage 5; lane 4, cells grown + 200 μM BPD; lane 5, cells grown + 300 μM BPD; lane 6, cells grown + 400 μM BPD.

with increasing iron restriction (i.e. 300, 400 μM BPD), there was increased TMBZ binding. A series of very lightly staining TMBZ bands might correspond to LPS.

Purification of hemin-binding protein

The 30 kDa *P. gingivalis* 381 hemin-binding protein was sequentially purified from the unheated cell envelopes (Fig. 2). In Fig. 2, lane 2, the cell envelope fraction contained numerous cell envelope proteins, including LPS-associated proteins. 1% CHAPS solubilization resulted in the removal of a large number of membrane proteins and associated LPS. The 30 kDa protein in Fig. 2, lane 3 was the major protein. The 30 kDa protein was isolated from the SDS-PAGE gels of the 1% CHAPS-soluble fraction (Fig. 2, lane 4). This protein was aggregated with proteins at 24 and 56 kDa. Heating of the isolated 30 kDa protein resulted in the appearance of the 24 kDa protein and several other minor and weakly staining proteins (Fig. 2, lane 5). The 24 kDa protein was isolated from the SDS-PAGE of this heated 30 kDa protein (Fig 2, lane 6). The resulting 24 kDa protein was isolated from the gel as a single protein band, with no contaminating proteins even when the gels were overloaded with large amounts of purified protein (data not shown).

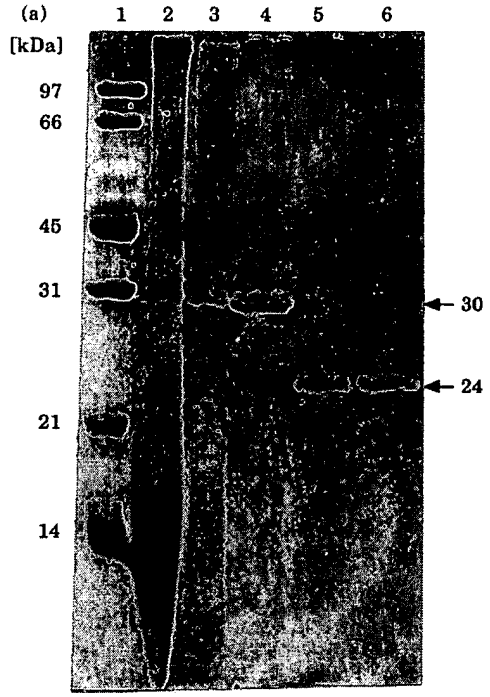

Fig. 2. SDS-PAGE analysis of the purification of the 30 kDa (unheated; 24 kDa heated) hemin-binding protein from *P. gingivalis* strain 381. Lane 1, low mol.wt. standards; lane 2, cell envelope fraction; lane 3, 1% CHAPS-soluble fraction; lane 4, isolation of 30 kDa protein; lane 5, 100°C, heated isolation 30 kDa protein; lane 6, purified 24 kDa protein.

Table 1 Cyanogen bromide fragmentation of 24 kDa hemin-binding protein from *Porphyromonas gingivalis* 381

Fragments	Amino acid sequence*
20 kDa	DQATSVPTDG(X)Y(X)TVD(X)KLGRITVK
17 kDa	GPDG(H)ZMEYEE
12 kDa	EYEEQGFSEVITGKKNAQGFAA(X)G(X)LEF(S)

*(X), unknown; (), assume to be correct amino acid.

N-terminal sequence analysis

The CNBr digestion of 24 kDa hemin-binding protein revealed at least three polypeptide bands (data not shown). Internal amino acid sequence analysis of one of these fragments (12 kDa) is seen in Table 1.

Discussion

Bacterial growth and membrane protein expression which are regulated by iron (hemin) have been reported in several microorganisms).[7–11] Many of these proteins have been implicated as functional components of iron (hemin) uptake systems

in these species. Although the requirement for hemin has been known for many years,[12] little is known about the mechanism(s) by which *P. gingivalis* binds and uptakes hemin into the cell. There have also been no reports of the purification and characterization of an actual hemin-binding protein from *P. gingivalis*.

Several Gram negative bacteria are known to utilize hemin as a sole source of iron. Hemin-binding proteins have been identified in several of these species, including *Shigella flexnerii*,[13] *Bacteroides fragilis*,[10] *Neisseria gonorrheae*,[14] *Hemophilus influenzae*,[15–17] *Treponema denticola*,[7] and *P. gingivalis*.[5,18] However, only few of these proteins have been purified and characterized. In the study presented here, we were able to establish that a 30 kDa (unheated) cell envelope associated protein from *P. gingivalis* strain 381 bound hemin and was stained with TMBZ. The expression of this protein appeared to be tightly regulated by the level of hemin(iron) in the growth medium.

Functionally, Omp 26 of Bramanti and Holt[18] and Omp 32 of Smalley *et al.*[5] appear similar with respect to hemin. Bramanti and Holt[18] were unable to sequence Omp 26 because of N-terminal blockage, and Smalley *et al.*[5] did not provide any sequence data for their Omp 32. Internal amino acid sequence analysis of the CNBr digested fragment and a search of GenBank for proteins with similar internal amino acid sequence to the 24 kDa protein revealed no significant similarities, and we consider the 30 kDa (heated 24 kDa) membrane protein to represent a newly described hemin binding protein from *P. gingivalis* strain 381. To our knowledge, the study described here is the first to identify, purify and biochemically characterize a hemin-binding protein from *P. gingivalis*. Work is in progress to further characterize the molecular structure of this protein.

Materials and methods

Bacterial strain and culture conditions. *P. gingivalis* 381 was grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) Mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1 μg/ml menadione and 5 μg/ml hemin. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24 h, equivalent to late exponential growth phase. For hemin restriction (i.e. hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e. 7.7 μM hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. Iron limitation was achieved by the addition of 100 to 400 μM of the iron-chelating compound, 2,2-bipyridyl (BPD., Sigma Chemical Co., St. Louis, MO), into liquid growth medium containing 7.7 μM hemin. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. Culture purity was assessed by Gram staining and plating to solid medium.

Cell envelope preparation. Cells were harvested by centrifugation at 12,000 × ***g***, for 20 min at 4°C, washed three times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2 mM each of phenylmethylsulfonyl fluoride (PMSF), benzamidine and Na-P-tosyl-L-lysine chloromethyl ketone (TLCK). Cell envelopes were prepared by French pressure cell disruption of whole cells in PBS (pH 7.2) by four 15,000 lb/in^2 disruption cycles. The cell envelopes were removed after low-speed (10,000 × ***g***, 30 min) and high-speed (2,000,000 × ***g***, 2 h) centrifugation. Protein concentration was determined using the bicinchoninic acid (BCA) assay of (Pierce, Lockford, IL).

Polyacryamide gel electrophoresis. The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli[19] was employed for determination of protein distribution, and lithium dodecyl sulfate (LDS) PAGE was used

for the TMBZ staining studies. 12% acrylamide separating gels were routinely used. All gels were run with a 4% acrylamide stacking gel in a vertical slab gel apparatus (Hoefer Scientific, San Francisco, CA). Proteins were visualized by Coomassie Brilliant Blue-R-250 stain (CBB). The hemin-associated peroxidase activity of the cell envelope protein was determined by tetramethylbenzidine (TMBZ, Sigma Chemical Co., St. Louis, MO) staining of LDS-PAGE gels. The TMBZ staining was carried out as described by Stugard *et al.*[13]

Purification of hemin-binding protein. Isolation of hemin-binding protein from *P. gingivalis* 381 cell envelope was accomplished by solubilization in the zwitterionic detergent 3 [(3-chloramidopropyl)-dimethyl-ammonio] 1 propane sulfonate (CHAPS; Pierce, Rockford, IL). Cell envelopes from *P. gingivalis* 381 (passage 5) were isolated as described above, and solubilized by the addition of CHAPS to a final concentration of 1% (v/v) and incubated at 37°C, 1 h. The resulting suspension was ultracentrifuged at 100,000 ***g*** for 1h to pellet insoluble material, and the CHAPS-soluble fraction was either used immediately for the purification of hemin-binding protein or stored at −20°C until used. The hemin-binding protein referred to as 'unheated 30 kDa' (see Results) from *P. gingivalis* 381 was purified from the CHAPS-soluble membrane fraction of hemin-starved passage five cells by 1D-SDS-PAGE through a 12% gel employing a preparative comb with one reference well. The elution protocol of Hager and Burgess[20] was used. Purity of the isolated protein was confirmed by 1D SDS-PAGE.

Cyanogen bromide digestion and N-terminal sequencing. Initial experiments revealed that the unheated 30 kDa protein did not transfer well to a polyvinylidine difluoride (PVDF) membrane (Pro Blot, Applied Biosystems, Foster City, CA). Heating the 30 kDa protein at temperatures above 70°C resulted in the modification of the protein to a molecular weight of 24 kDa. In this heated, denatured condition the protein transferred quantitatively, and was used for N-terminal amino acid analysis. Attempts to directly sequence the N-terminus of the 24 kDa protein were unsuccessful because of a blocked N-terminus. Therefore, sequences were determined after cyanogen bromide (CNBr) digestion.

Cleavage by CNBr was carried out on the acetone precipitated 24 kDa protein. Several crystals of CNBr were added to 100 μl of 70% formic acid and swirled to dissolve. 50 μl of this solution was added to the acetone precipitated sample and allowed to proceed in the dark at room temperature, 16 h. The digest was dried under an N_2 stream and reduced to dryness in a SpeedVac SC 100 (Savant). The dried digest was dissolved in 1 × treatment buffer for SDS-PAGE, heated at 100°C for 5 min, electrophoresed through an exponential gradient gel (7.5 to 20%), and electroblotted to a PVDF membrane at 100 mA, 4 h. After transfer, the ProBlot membrane was removed from the transblotting sandwich and rinsed with deionized water. Protein bands on the ProBlot membrane was visualized by Amido black staining. The protein bands were excised from the dried membrane, and its N-terminal amino acid sequence was determined with an Applied Biosystems (Foster City, CA) Model 477A gas–liquid phase sequenator coupled to an on line high-performance liquid chromatography model 120A analyser.

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Porphyrin-Mediated Binding to Hemoglobin by the HA2 Domain of Cysteine Proteinases (Gingipains) and Hemagglutinins from the Periodontal Pathogen *Porphyromonas gingivalis*

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Heme binding and uptake are considered fundamental to the growth and virulence of the gram-negative periodontal pathogen *Porphyromonas gingivalis*. We therefore examined the potential role of the dominant *P. gingivalis* cysteine proteinases (gingipains) in the acquisition of heme from the environment. A recombinant hemoglobin-binding domain that is conserved between two predominant gingipains (domain HA2) demonstrated tight binding to hemin (K_d = 16 nM), and binding was inhibited by iron-free protoporphyrin IX (K_i = 2.5 μM). Hemoglobin binding to the gingipains and the recombinant HA2 (rHA2) domain (K_d = 2.1 nM) was also inhibited by protoporphyrin IX (K_i = 10 μM), demonstrating an essential interaction between the HA2 domain and the heme moiety in hemoglobin binding. Binding of rHA2 with either hemin, protoporphyrin IX, or hematoporphyrin was abolished by establishing covalent linkage of the protoporphyrin propionic acid side chains to fixed amines, demonstrating specific and directed binding of rHA2 to these protoporphyrins. A monoclonal antibody which recognizes a peptide epitope within the HA2 domain was employed to demonstrate that HA2-associated hemoglobin-binding activity was expressed and released by *P. gingivalis* cells in a batch culture, in parallel with proteinase activity. Cysteine proteinases from *P. gingivalis* appear to be multidomain proteins with functions for hemagglutination, erythrocyte lysis, proteolysis, and heme binding, as demonstrated here. Detailed understanding of the biochemical pathways for heme acquisition in *P. gingivalis* may allow precise targeting of this critical metabolic aspect for periodontal disease prevention.

Evidence for the potential importance of cysteine proteinases from *Porphyromonas gingivalis* in periodontal disease pathology is increasing. Periodontal disease affects the majority of adults to some degree and may be associated with significant systemic morbidity (2, 46), including dental infection and loss of teeth (36). *P. gingivalis* is implicated as an important periodontal pathogen by its high incidence and relative levels in human disease (1, 11) and by its virulence in monoinfected animals (14, 15). Virulence of *P. gingivalis* has been attributed to several components of the microorganism, including fimbriae (25, 37), short-chain volatile acids (12, 65), lipopolysaccharide (26, 58), collagenase activity (3, 39), and noncollagenolytic cysteine proteinase activity (8, 10, 54).

Cysteine proteinase activity may affect the remodeling of matrix proteins and disrupt the immune response by stimulating the collagen-degrading activity of host cells (8, 10, 62), degrading fibronectin (34), inactivating gamma interferon (68) and interleukins (6, 17), interfering with the complement cascade (63, 67), and degrading immunoglobulins (16, 52). Also, clotting and vascular permeability mechanisms may be disturbed (27, 28, 54), fibrinogen may be degraded (33, 54), and erythrocytes may be agglutinated and lysed (44, 56) by cysteine proteinase activity, possibly for the acquisition of metabolically necessary iron, heme, or porphyrin from hemoglobin. Numerous different *P. gingivalis* cysteine proteinases described in several reports have been demonstrated to be antigenically related (9, 47, 48) and the products of three related genes (41, 51). This unique family of enzymes, named gingipains, has two major gene products, Arg-gingipain-1 (RGP-1) and Lys-gingipain (KGP) (41), which prefer proteinacious substrates with an arginine or lysine in the P1 position, respectively.

Bacterial cysteine proteinase activity has been demonstrated within diseased periodontal pockets (13, 20), and epitopes of gingipains are detectable in clinical plaque samples from patients with adult periodontitis (unpublished data), so the gingipains are likely to be clinically relevant. The gingipains are expressed on the outer membrane of *P. gingivalis* and may also be released with vesicles or as soluble proteins (9, 18, 24). Gingipains have been suggested to account for up to 85% of trypsin-like proteolytic activity in a *P. gingivalis* culture (49), and under certain growth conditions in vitro, these enzymes can accumulate to become the most abundant *P. gingivalis* proteins in a culture (9).

The catalytic domains of RGP-1 and KGP constitute approximately one-third of the translated protein products. The remaining two-thirds of these two gingipain molecules consist of four COOH-terminal domains (HA1 to HA4) which are highly homologous between these two predominant gingipains (Fig. 1). These noncatalytic COOH-terminal domains were originally named hemagglutinin (HA) domains because at least one was thought to participate in hemagglutination (47). They may each be separated posttranslationally from the catalytic domain and from one another, presumably through au-

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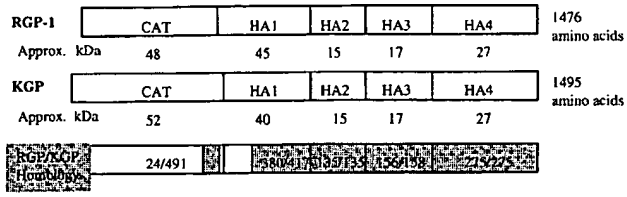

FIG. 1. Domain structure and homologies between the gingipains RGP-1 and KGP. CAT represents the putative catalytic domain. Shaded areas represent regions of >98% amino acid identity between the two gingipains. Each fraction represents the degree of identity for each RGP-1 domain. Approx. kDa, approximate molecular mass in kilodaltons.

tolysis some time after logarithmic growth in vitro (9, 59). The functions of the first, third, and fourth HA domains are unknown. The second HA domain (HA2) has recently been implicated in hemoglobin binding (19, 43). Because all of the domains of the gingipains are found together predominately in loose, noncovalent associations with one another after hydrolytic separation (9, 59), the gingipains appear to be multifunctional proteins for aggregation of erythrocytes and then lysing of these cells to obtain hemoglobin for the acquisition of iron, heme, or porphyrin.

P. gingivalis (formerly *Bacteroides* sp.) can utilize inorganic iron, free or protein-associated heme, or organic iron sources such as transferrin (5). Several investigators have previously shown that *P. gingivalis* binds to and internalizes hemin with various affinities and at various rates (4, 21, 53, 57, 60, 64). These earlier reports suggest that there are at least two heme-binding proteins of *P. gingivalis* with different affinities for hemin which may respond to environmental changes by rapidly changing their position or associations within the outer membrane.

Hemin binding and uptake appear to be related to the regulation of proteinase and fimbriae expression and to vesicle formation (7, 38, 40) and were recently proposed to establish an antioxidative shield for protection from oxidative radicals (61). Binding of protoporphyrin IX in *P. gingivalis* was also implied by competition with labelled hemin (4, 64), and protoporphyrin IX was reported to support growth (53). Protoporphyrin IX limitation was shown to be coordinated with phenotypic expression of proteinase activity (42). Hemin binding by *P. gingivalis* may therefore represent a capacity for protoporphyrin binding.

Recently, Nakayama et al. have isolated a hemoglobin-binding protein associated with the outer membrane of *P. gingivalis* and identified this protein as one homologous with the HA2 domain of the gingipains (43). In that report, adsorption of hemoglobin to whole *P. gingivalis* cells was associated with the presence of the HA2 domain. Also, hemin accumulation within the *P. gingivalis* cells was shown to be dependent on functional expression of KGP (45). The HA2 gingipain domain may therefore function as a hemoglobin-binding domain in *P. gingivalis*.

Understanding the molecular and biochemical mechanisms involved in key regulatory pathways is paramount in developing strategies for control of disease. In this study, we obtained evidence, by using a monoclonal antibody (MAb) which recognizes the hemoglobin-binding (HA2) domain of *P. gingivalis* cysteine proteinases, that the HA2 domain can bind to hemoglobin primarily and specifically through a portion of the heme moiety that is surface exposed in the hemoglobin structure. We also found that the unique epitope of MAb 5A1 within this heme-binding domain was expressed in parallel with hemoglobin-binding activity and proteinase activity in cellular and cell-free culture fractions of *P. gingivalis*.

MATERIALS AND METHODS

RGP-1 and KGP isolation. Polydomain RGP-1 and KGP were isolated and characterized as previously described (68) by arginine-Sepharose affinity chromatography of detergent-extracted *P. gingivalis* ATCC 33277 cells. Alternatively, polydomain RGP-1 and KGP were isolated as previously described (9) by arginine-Sepharose affinity chromatography from cell-free supernatant of a 10-day *P. gingivalis* batch culture.

Enzyme activity assays. The proteinase activities of *P. gingivalis* culture fractions were measured by using the substrates *N-tert*-butoxycarbonyl-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin or *N-tert*-butoxycarbonyl-Glu-Lys-Lys-7-amido-4-methylcoumarin at 30°C in Tris buffer without added reducing agents. Substrate hydrolysis was monitored over time by measuring A_{460} with a 380-nm excitation beam on a Perkin-Elmer LS 50B luminescence spectrophotometer.

Development of MAbs 5A1 and IIB2. Antigingipain MAbs 5A1 and IIB2 were prepared in mice as previously described (9).

Expression and purification of recombinant HA2 (rHA2). Forward and reverse primers (AACCTGCAGCGCGCAGACTTCACGG and GGAAGCCAA TGGCGCCAAAAGATCTAGT) were designed to amplify the HA2 domain from the *P. gingivalis* RGP-1 proteinase gene (accession no. U15282). Restriction sites for *Pst*I and *Bgl*II were designed into the 5′ ends of the primers to facilitate cloning. The digested PCR product was ligated into the QIAexpressionist type III construct providing a six-His tag at the COOH terminus (Qiagen Corp.). Transformation of the ligated construct was performed by electroporation into *Escherichia coli* NM522 cells. *E. coli* cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. Cells were harvested and resuspended to 5 ml/g (wet weight) in buffer A (8 M urea, 0.1 mM NaH_2PO_4, 0.01 mM Tris-HCl, pH 7.9). The cells were stirred for 2 h at room temperature, taking care to avoid foaming. This cell lysate was subjected to centrifugation at 31,000 × *g* for 30 min at room temperature to pellet the cellular debris, and then the supernatant was subjected to ultracentrifugation at 130,000 × *g* for 2 h. The clarified lysate was loaded onto a nickel-nitrilotriacetic acid column (Qiagen Corp.), pre-equilibrated with buffer A. The nickel-nitrilotriacetic acid column was washed with buffer A until the baseline was reached. The protein was refolded on this column by running a linear gradient of urea from 8 to 0 M in 20 mM Tris-HCl–500 mM NaCl–10% glycerol (pH 7.9). The protein was then eluted with 50 mM Tris-HCl–500 mM NaCl–10% glycerol–250 mM imidazole (pH 7.9). The eluant was diluted 100-fold in 50 mM sodium acetate buffer (pH 5.5) and applied to a hemoglobin-agarose column pre-equilibrated with the dilution buffer. After loading, the column was washed with the same buffer until the baseline was reached and then the hemoglobin-binding protein was eluted with 50 mM Tris-HCl (pH 9). Protein concentrations were determined by Coomassie dye binding using bovine serum albumin as the standard.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using 12% gels with 4% stackers by the method of Laemmli (32). All samples were diluted with SDS sample buffer before electrophoresis with (reducing) or without 2-mercaptoethanol. Western blotting was performed by the method of Towbin et al. (66), and proteins were transferred from the gels to polyvinylidene difluoride (PVDF) paper (Bio-Rad) with 300 mA for 1 h. Blots were blocked with 0.1% bovine serum albumin in 20 mM Tris-HCl with 500 mM NaCl containing 0.1% Tween 20 (TBS/Tween). An alkaline phosphatase (AP) conjugate of rabbit anti-mouse immunoglobulin G (Dako Corp.) was used as a secondary antibody. Blots were washed with TBS/Tween between antibody applications. The substrate for AP was nitroblue tetrazolium in excess with 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), and color was developed in 5 mM Tris (pH 9.5).

NH_2-terminal amino acid sequencing of proteins resolved by SDS-PAGE was performed as previously described (9).

ELISA. Enzyme-linked immunosorbant assays (ELISA) were performed in polystyrene microtiter wells. Proteins were used to coat the surfaces in 2.7 mM KCl–1.5 mM KH_2PO_4–137 mM NaCl–8.1 mM Na_2HPO_4 (PBS) with 10 mM sodium azide (PBS/N_3). All wells were blocked and washed in PBS with 0.1% Tween 20 (PBS/Tween). Primary murine antibodies were applied in PBS/Tween at a concentration of 0.5 μg/ml for at least 1 h. Secondary goat anti-mouse antibodies conjugated with AP (Dako Corp.) were applied at a concentration of 1.1 μg/ml for 30 min, and then AP activity was monitored at 414 nm by hydrolysis of the substrate 4-nitrophenylphosphate (Boehringer GmbH, Mannheim, Germany) in 5 mM Tris (pH 9.5) by using a Titertek Twinreader PLUS photometer (absorbance maximum of 3.0 ELISA units). Mean apparent dissociation constants (K_ds) were derived by solid-phase ELISA as previously described (50) and are accompanied by standard errors of the means.

Ligand-binding assay. The ligand-binding assay was a variant of the ELISA in which the ligand (i.e., hemin or hemoglobin) that had been used to coat the wells in PBS/N_3 was subsequently allowed to bind to a second ligand-binding protein (i.e., rHA2 or gingipains) in PBS/Tween. The ligand-binding protein was then detected with MAb 5A1 or IIB2, followed by a rabbit anti-mouse AP conjugate, and developed as already described for ELISA. Bovine hemoglobin was used in these experiments. Hemin was from stock solutions dissolved in 0.1 N NaOH,

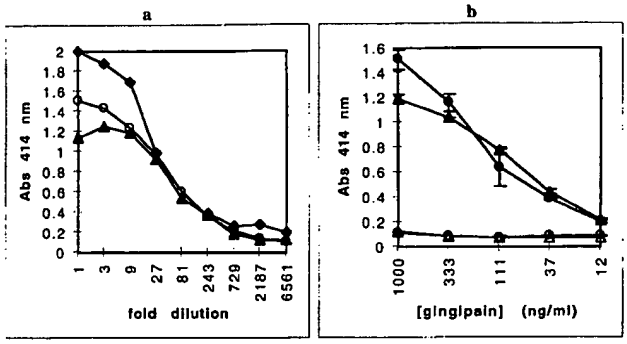

FIG. 2. Hemoglobin binding by rHA2, RGP-1, and KGP. (a) Microtiter wells were coated with hemoglobin and then incubated with threefold dilutions of purified rHA2 at 2.5 μg/ml (◆), RGP-1 at 5 μg/ml (○), or KGP at 5 μg/ml (▲). Association of rHA2 with hemoglobin was measured with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. (b) Hemoglobin binding by native, but not denatured, gingipains. Wells were coated with hemoglobin and then incubated overnight with dilutions of either RGP-1 (●), KGP (▲), RGP-1 denatured by boiling (○), or KGP denatured by boiling (△). For this experiment, native or denatured gingipains that bound to hemoglobin were recognized by MAb IIB2, which specifically detects both native and denatured gingipains. Primary antibody IIB2 was followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of three separate experiments. Abs, absorbance.

and although the NaOH would replace the chloride ion of hemin with a hydroxylate ion (hematin), the term hemin will be used for this compound throughout this report. The K_d and apparent inhibition constant (K_i) for ligand binding were derived as previously described (50) in these assays by using serial dilutions of the ligand-binding protein or competitor, respectively, with even amounts of coated ligand. The reported results are means accompanied by the standard errors of the means.

Peptide synthesis. Peptides were synthesized by Chiron Mimotopes with terminal amines and carboxylic acids. The peptide 1 sequence was ALNPDNYLISKDVTG, and the peptide 2 sequence was GEAPAEWTTIDADGDGQGWL.

Materials. All chemicals and compounds were purchased from Sigma unless otherwise specified.

Statistics. Statistical differences between measurements of the gingipains and rHA2 were determined with one-tailed Student *t* tests.

RESULTS

The polydomain RGP-1 and KGP isolated from 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-extracted *P. gingivalis* cells possessed SDS-PAGE profiles, NH_2-terminal sequences, proteolytic activities, and inhibition profiles characteristic of gingipain-like molecules previously described by us (9, 68) and others (3, 47, 54) (data not shown).

The HA2 domain was cloned, expressed, and purified as a six-His tag fusion. Nucleic acid and NH_2-terminal amino acid sequencing verified the identities of the clone and the expressed protein, respectively, as the HA2 domain of RGP-1 (data not shown).

Hemoglobin is bound by rHA2 and by native but not denatured RGP-1 and KGP. In the solid-phase ligand-binding assay, rHA2, RGP-1, and KGP each bound to hemoglobin (Fig. 2a). As MAb 5A1 was used to detect rHA2 bound to hemoglobin and did not interfere with this binding, it was evident that the epitope for MAb 5A1 within the HA2 domain was separate from the hemoglobin-binding site of HA2. The hemoglobin-binding affinities of rHA2, RGP-1, and KGP (K_d = 2.1 ± 0.6 nM) were similar (P = 0.24), and the binding curves of neither rHA2 nor the gingipains were indicative of multisite binding (Fig. 2a). High-affinity binding to hemoglobin at a single site within only the HA2 domain of both native RGP-1 and KGP is sufficient to account for these observations. The binding site for hemoglobin within the gingipains appeared to be associated with a higher-order protein structure, since denaturation of RGP-1 and KGP by boiling effectively eliminated their ability to bind hemoglobin (Fig. 2b).

Hemoglobin binding of the HA2 domain is mediated through the heme moiety. To begin characterizing the binding between rHA2 and hemoglobin, we examined the binding between rHA2 and hemin, as well as binding to hemoglobin degraded by proteinase K. rHA2 bound not only to wells coated with hemoglobin but also to wells coated with hemin or with proteolytically degraded hemoglobin (Fig. 3a). Binding of the rHA2 domain to hemin-coated wells was approximately eightfold weaker than binding to hemoglobin in solid-phase assays (K_d = 16 ± 1 nM) (Fig. 3b).

The HA2 domain binds the porphyrin ring structure. To dissect the binding of the rHA2 domain to hemin, the K_is of iron-free protoporphyrin IX in solution phase competition assays were determined. By using the standard ligand-binding assay described herein, rHA2 or the gingipains were preincubated with dilutions of protoporphyrin IX and then allowed to bind to the hemin-coated wells. Binding of the gingipains or rHA2 to hemin was inhibited by the addition of protoporphyrin IX (K_i = 2.5 ± 0.3 μM) (Fig. 4a). The apparent K_i values of rHA2 and the gingipains were similar (P = 0.42). These data indicated that binding of rHA2 or the gingipains to hemin was specific for some aspect of the protoporphyrin ring. Importantly, binding of rHA2 or the gingipains to hemoglobin was also inhibited by protoporphyrin IX (Fig. 4b) (K_i = 10 ± 2 μM) and preincubation with the protoporphyrin effectively eliminated binding to hemoglobin.

Directed protoporphyrin binding by rHA2. Examination of the hemoglobin crystal structure indicated that only the region of the heme moiety possessing the propionate functional groups (Fig. 5) would be exposed for possible protein-protein contact. We therefore reasoned that blocking access to the acidic region of protoporphyrin molecules would have an effect on rHA2 binding and allow more specific characterization of binding between the HA2 domain and the porphyrin ring. In a modification of the ligand-binding assay system described

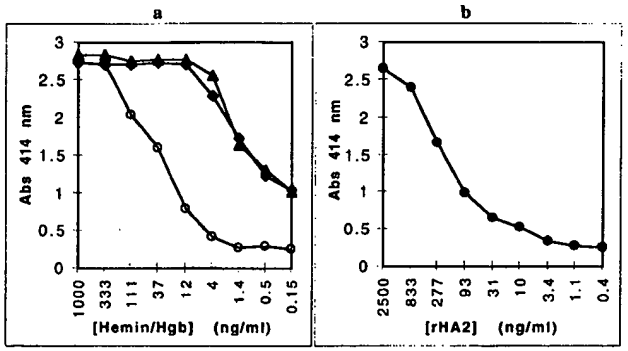

FIG. 3. Binding of the HA2 domain to the heme moiety. (a) Binding of rHA2 to dilutions of hemin (◆), hemoglobin (Hgb) (○), or hemoglobin degraded by proteinase K (▲). Microtiter wells were coated with dilutions of samples, and then overnight binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. The absence of contaminating protein within 90 μg of the hemin preparation and the absence of nondegraded subunits of hemoglobin remaining after proteinase K treatment were verified by SDS-PAGE (data not shown). (b) Binding of rHA2 to hemin. Microtiter wells were coated with hemin, and overnight binding of rHA2 dilutions was detected with MAb 5A1 as described above. These data are representative of two separate experiments. Abs, absorbance.

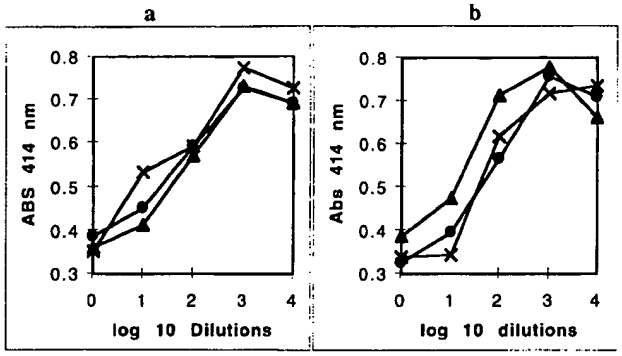

FIG. 4. Inhibition of hemin or hemoglobin binding. Microtiter wells were coated overnight with hemin (a) or hemoglobin (b). rHA2 in *E. coli* lysate (100-fold dilution) (×), RGP-1 at 65 ng/ml (●) or KGP at 65 ng/ml (▲) was preincubated with dilutions of 300 μM protoporphyrin IX for 1 h and then transferred to the ligand-coated plates for overnight incubation. Binding of rHA2 or the gingipains to coated wells was detected with MAb 5A1 or IIB2, respectively, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody. These data are representative of two separate experiments. The absence of contaminating protein in a 90-μg protoporphyrin IX preparation was verified by SDS-PAGE and Coomassie dye binding (data not shown). ABS, absorbance.

above, surfaces were first coated with ethylene diamine to provide fixed, free, primary amines for carbodiimide linkage of carboxylic acid groups. Hemin, protoporphyrin IX, and hematoporphyrin bound to wells coated with ethylene diamine with or without carbodiimide treatment, as determined by A_{414} measurement (Fig. 5, striped bars). rHA2 binding to the carbodiimide-treated porphyrins in the wells was almost eliminated, however, compared to the relatively greater association of rHA2 with the nonderivatized porphyrins (Fig. 5, solid bars). These data indicated that the rHA2 domain specifically recognized the three porphyrin compounds in the region of the propionic acid groups, as we were able to block rHA2 binding by directionally attaching the carboxylic acids of hemin, protoporphyrin IX, or hematoporphyrin to fixed amines. Since the heme moiety within hemoglobin is almost identical to these porphyrin molecules, the data suggested that the heme moiety of hemoglobin was bound by rHA2 and by the HA2 domain of the gingipains in a similar, directed, high-affinity manner.

The MAb 5A1 epitope is recognized in the rHA2 domain and in denatured, but not native, RGP-1 and KGP. In ELISA, MAb 5A1 bound to rHA2 with high affinity (K_d = 2.2 × 10^{-10} ± 0.5 × 10^{-10} M) (Fig. 6a). MAb 5A1 also bound to denatured RGP-1 and KGP but did not bind to the native gingipains isolated from CHAPS-extracted *P. gingivalis* cells (Fig. 6b). Soluble high-molecular-weight aggregates of gingipain domains isolated from the cell-free fraction of a *P. gingivalis* batch culture by arginine-Sepharose affinity chromatography (9) were, however, recognized by MAb 5A1 (K_d = 1.7 × 10^{-10} ± 0.6 × 10^{-10} M) (Fig. 6c). The similarity of the dissociation constants (P = 0.36) and binding curves suggested that MAb 5A1 recognized the same HA2 epitope in these polydomain gingipains as in rHA2.

The MAb 5A1 epitope is represented by an amino acid sequence within the HA2 gingipain domain. By use of linear synthetic peptides, the epitope of MAb 5A1 was determined to be associated with the peptide ALNPDNYLISKDVTG (K_d = 3.8 nM), which represents amino acids 1215 to 1229 of translated KGP within the HA2 domain (Fig. 7, peptide 1). Dot blot analysis on a PVDF membrane confirmed the unique immunoreactivity of this peptide with MAb 5A1 (data not shown). A search of the SwissProt database for the linear sequence of peptide 1 or of the GenBank database by using the deduced nucleic acid sequence of this epitope resulted in no molecules with perfect homology to the peptide other than the gingipains and HagA, a large HA with regions of identity to the entire HA2 domain.

Correlation of HA2 domain immunoreactivity with hemoglobin binding in a *P. gingivalis* culture. Detection of the HA2 epitope with MAb 5A1 in unfractionated *P. gingivalis* samples was correlated with hemoglobin binding. Because proteinase activity and gingipain expression have been shown to progressively change during the course of an extended *P. gingivalis* batch culture (9), we examined cell-associated and extracellular fractions during 8 days of culture. Both Arg- and Lys-

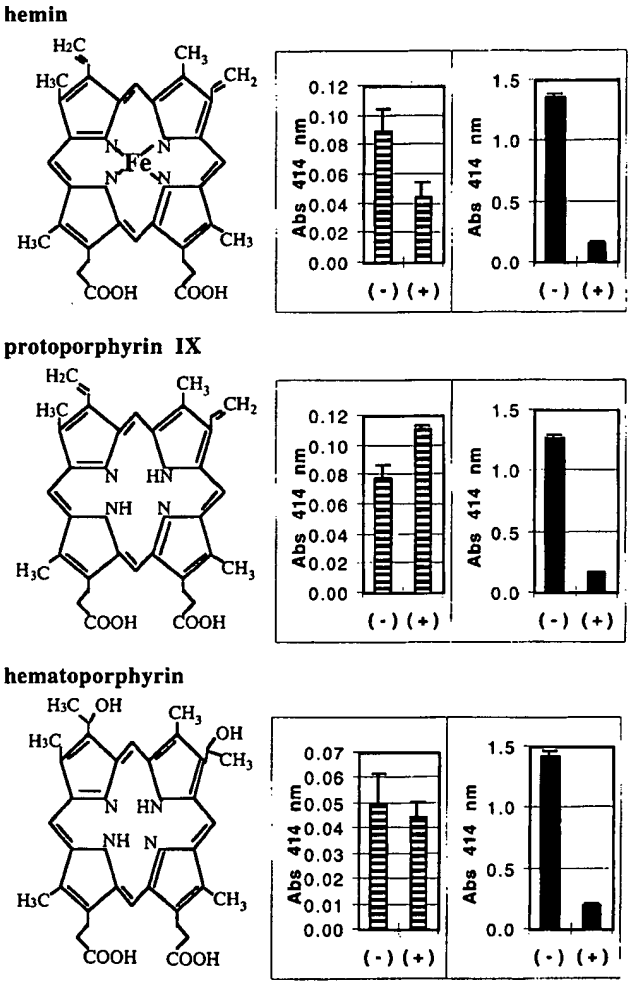

FIG. 5. Directed porphyrin binding by rHA2. Microtiter wells were coated with 100 mM ethylene diamine (pH 4.7) and then incubated with hemin, protoporphyrin IX, or hematoporphyrin at 90 μg/ml overnight in 50% dimethyl formamide in the presence (+) or absence (−) of 10 mM carbodiimide. Wells were washed four times with water, and then the amount of porphyrin bound to the wells was determined by measuring absorbance (Abs) at 414 nm (striped bars). Wells were blocked with PBS/Tween and then incubated with rHA2 at 125 ng/ml overnight. Binding of rHA2 to coated wells was detected with MAb 5A1, followed by substrate development after binding of a secondary anti-mouse AP-conjugated antibody (solid bars). Error bars represent standard deviations of absorbance measurements. Diagrams of the chemical structures of hemin, protoporphyrin IX, and hematoporphyrin are presented adjacent to the corresponding data.

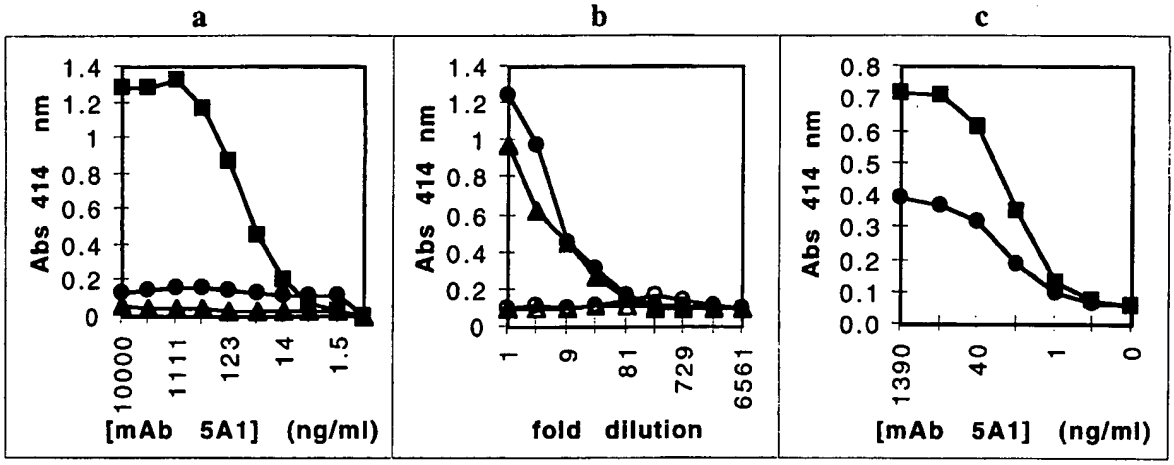

FIG. 6. Measurement of high-affinity binding of MAb 5A1 with rHA2, denatured but not native gingipains, and gingipains from the culture supernatant. (a) RGP-1 (●), KGP (▲), or rHA2 in crude *E. coli* lysate (■) was used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. (b) Dilutions of RGP-1 (○), KGP (△), or heat-denatured RGP-1 (●) or KGP (▲) were used to coat microtiter wells with threefold dilutions from 10 μg/ml and then incubated with MAb 5A1. (c) Purified rHA2 (■) or purified high-molecular-weight aggregates of gingipain domains isolated from culture supernatant (●) were used to coat microtiter wells and incubated with serial dilutions of MAb 5A1. These data are representative of three separate experiments. Abs, absorbance.

specific proteinase activities of the *P. gingivalis* cells peaked near day 3 of culture (Fig. 8a and b, triangles). Proteinase activities of the cell-free culture supernatants steadily rose throughout the culture period (Fig. 8a and b, squares).

Immunoreactive protein in the cell-free conditioned culture medium detected with MAb 5A1 steadily accumulated throughout the 8-day culture period, similar to proteolytic activity (Fig. 8c, open squares). Immunoreactive protein associated with hemoglobin binding in this supernatant fraction also increased steadily throughout the extended culture in a parallel manner (Fig. 8c, closed squares). In the cellular fraction of the *P. gingivalis* culture, expression of immunoreactive protein increased early during the culture period with a peak near day 3 followed by a slight decrease and then an increase to peak levels again by day 7, similar to the proteolytic activity of this fraction (Fig. 8c, open triangles). Immunoreactive protein associated with hemoglobin binding in the cellular fraction followed a parallel pattern of expression (Fig. 8c, closed triangles). These data demonstrated that detection of protein immunoreactive with MAb 5A1 in crude cellular and extracellular fractions of a *P. gingivalis* culture was directly associated with hemoglobin binding, suggesting that MAb 5A1 specifically recognized the hemoglobin-binding HA2 domain within the *P. gingivalis* culture. Also, the data demonstrated a profile of HA2 domain expression and hemoglobin-binding activity similar to the profile of cellular and extracellular proteolytic activity expressed by *P. gingivalis*.

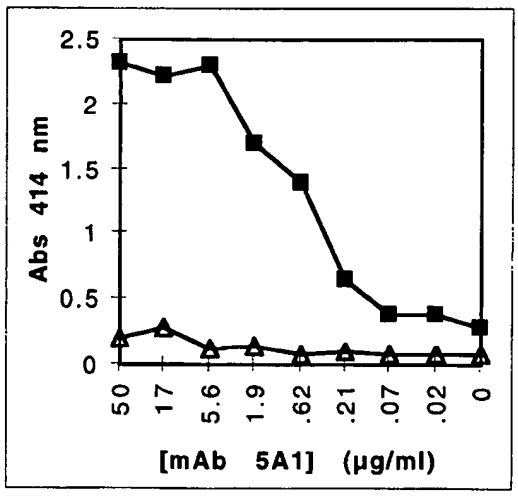

FIG. 7. Immunoreactivity of synthetic peptides with MAb 5A1. ELISA demonstrating selective immunoreactivity of MAb 5A1 with peptide 1. Peptide 1 (■) or 2 (△) was used to coat microtiter plates at a concentration of 5 μg/ml, incubated overnight, and then incubated with dilutions of MAb 5A1. These data are representative of two separate experiments. Abs, absorbance.

DISCUSSION

Control of *P. gingivalis* growth to prevent periodontal pathology might be achieved by interference with one or more pathways for obtaining heme. To this end, we have reported on a MAb which recognizes an epitope within the hemoglobin-binding domain of the abundant *P. gingivalis* cysteine proteinases, named gingipains, and demonstrated increasing levels of this HA2 domain associated with hemoglobin binding and proteinase activity in an extended *P. gingivalis* culture. Further, we have characterized the binding between the HA2 domain and hemoglobin, suggesting that binding is mediated in large part by specific recognition of the porphyrin ring of the heme moiety within hemoglobin.

The hemoglobin-binding affinities of RGP-1, KGP, and the HA2 domain measured in our experiments were similar. Also, binding curves for these interactions were typical of single-site binding, which is consistent with the idea that the HA2 domain of the cell-derived gingipains is solely responsible for hemoglobin binding. The similarity of the inhibition profiles for the gingipains to that of rHA2 further suggested that mediation of gingipain binding to heme was through only the HA2 domain. These data do not, however, rule out other possible heme-binding sites in the gingipains with affinity identical to that of HA2.

Hemoglobin binding by the separated catalytic domain of KGP was recently demonstrated (31). Our data, obtained by using polydomain gingipains, did not provide evidence for this

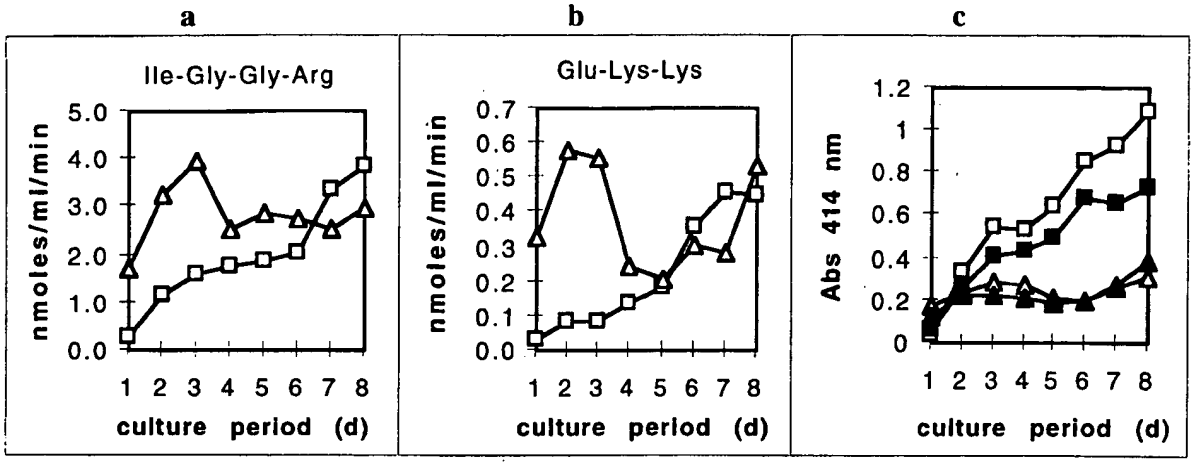

FIG. 8. Expression of HA2-related immunoreactive hemoglobin-binding protein from *P. gingivalis*. Aliquots of *P. gingivalis* culture medium were removed daily during a period of 8 days (d), immediately separated into a cell pellet and culture supernatant, and then frozen until use. The OD_{660} and purity of the culture were measured daily. The cell pellets were dispersed evenly into 1 ml of PBS/N_3. (a and b) Arg- and Lys-specific proteinase activities, respectively, of the cell-free culture supernatant (□) and cellular fraction (△) were measured as described in Materials and Methods. Measurements of the cellular fractions were normalized to the culture densities (OD_{660}) recorded daily. (c) The HA2 domain (1/243 dilution; □) and the HA2 domain associated with hemoglobin binding (1/81 dilution; ■) in culture supernatants were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. In *P. gingivalis* whole-cell fractions, the HA2 domain (1/243 dilution; △) and the HA2 domain associated with hemoglobin binding (1/9 dilution; ▲) were measured by ELISA and a ligand-binding assay, respectively, as described in Materials and Methods. Measurements of the cell-associated fractions were normalized to the culture densities (OD_{660}) recorded daily. The corresponding background immunoreactivity with murine anti-human CD-19 immunoglobulin G was subtracted from each measurement. These data are representative of two separate experiments in which the patterns of expression were similar. Abs, absorbance.

second hemoglobin-binding site. It is likely, however, that separated domains of the gingipains behave differently than when associated either noncovalently or within a single polydomain polypeptide. The inability of MAbs which recognize either isolated gingipain domains or peptides to recognize the larger polydomain gingipains of cells exemplifies this potential (this report and reference 22).

Apparent dissociation constants in the nanomolar range represented significantly tighter binding of the HA2 domain to hemoglobin than previously reported (43). Further, this relatively tight binding in our experiments was measured at a nearly neutral pH and not at the pH maximum for binding of 5.5 reported earlier. Differences in experimental systems for measuring binding may account for this discrepancy.

Protoporphyrin IX inhibited binding of rHA2 to hemin. Also, protoporphyrin IX and hemin did not differ statistically in the ability to inhibit the binding of rHA2 to hemin (data not shown). This indicated that the sequestering of porphyrin by HA2 functioned independently of iron. The side chain groups of the porphyrin also did not appear to determine HA2 binding. Hematoporphyrin differs from protoporphyrin IX only by the hydroxylation of the two side chain ethylene groups. These groups are located opposite the positions of propionate groups across the plane of the porphyrin. As the binding to HA2 of these two porphyrins was comparably strong (Fig. 5), it can be concluded that HA2 binding was insensitive to the nature of the chemical groups attached at these positions. This contrasts with the blocking of rHA2 binding in both hematoporphyrin and protoporphyrin IX by directional attachment through chemical modification of the propionate groups.

The iron chelator 2,2'-dipyridal at a concentration of 2 mM also inhibited the binding of rHA2 to hemin, although the K_i of the dipyridal was 200-fold higher than the K_i of protoporphyrin IX (data not shown). This may indicate that rHA2 also had some weak interaction with the iron, but direct steric interference by the dipyridal in the absence of direct iron binding by rHA2 could also be considered.

Binding of hemin by the rHA2 domain was eightfold weaker than that of hemoglobin, although it would be expected to be similar if binding of the HA2 domain to hemoglobin occurred solely through the porphyrin ring of the heme ligand. Competition experiments demonstrated that protoporphyrin IX also inhibited hemoglobin binding, although it was approximately fourfold less competitive than in hemin-binding assays. A portion of the hemoglobin polypeptide may, therefore, contribute to the interaction of HA2 with hemoglobin in a cooperative manner. Because protoporphyrin IX alone completely blocked the interactions between rHA2 or the gingipains and hemoglobin, however, binding between the HA2 domain and the heme moiety must have been essential for the maintenance of this cooperative hemoglobin binding. Alternatively, the weaker binding of rHA2 with hemin in these experiments might also be due to the possibility that iron-protoporphyrins in solution can dimerize, ruffle, or associate differently than when bound to hemoglobin (23, 29, 55). Further, the HA2-binding region of the relatively smaller hemin ligand when bound directly to a surface may be less sterically accessible to the HA2 domain than when heme is presented and supported as part of a large globular protein where the propionate groups and the adjoining rim of the porphyrin ring protrude slightly beyond the surface of the protein (35).

Gingipains recovered from the culture supernatant subsequent to the first day of growth were previously shown to consist of noncovalently aggregated lower-molecular-weight domain fragments of the gingipains (9, 59). Although MAb 5A1 did not recognize native gingipains purified from solubilized *P. gingivalis* cells, MAb 5A1 did detect gingipain domain aggregates purified from the culture supernatant. This is not surprising, considering that the antibodies were made against the domain fragments of these gingipains (9), and it demonstrates potential differences between high-molecular-weight gingipains recovered by various means.

It is not known whether the HA2 domain was recognized in our cultures as a separate domain, as implicated by the isola-

tion of the separate HA2 domain from envelope fractions (19), or whether the HA2 domain was part of a polydomain complex of gingipain fragments or derived from the *hagA* gene product. Since the gingipains would be required for hydrolytic release of the HA2 domain from the *hagA* gene product, as well as from the gingipains themselves (43, 45), analysis of porphyrin binding in *hagA* knockout strains of *P. gingivalis* is needed to address this question. Our data demonstrated that the presence of the HA2 domain released by the cells paralleled proteinase activity, as well as hemoglobin-binding activity, suggesting that the hemoglobin-binding HA2 domain was derived from the gingipains. Although these data do not directly implicate the HA2 domain in iron, heme, or porphyrin acquisition by the *P. gingivalis* organism, the HA2 domain was associated with hemoglobin binding and could be considered a specific target for interference with heme acquisition by *P. gingivalis*. An HA2-specific antibody which blocks HA2 binding to heme or hemoglobin might be useful in dissecting the role of this porphyrin-binding domain in whole-cell metabolism and virulence.

Hemagglutination was the original function ascribed to the four COOH-terminal domains of the gingipains (47). Although the HA2 domain functions as a porphyrin-binding domain, it might, in addition, participate in hemagglutination. The separate rHA2 domain, at a concentration of 2 μg/ml, did not agglutinate erythrocytes, however, and MAb 5A1, which bound to the HA2 domain, did not inhibit the hemagglutination capacity of whole *P. gingivalis* cells (data not shown). We are currently investigating the functions of each gingipain HA domain.

Sequence analysis and trypsin susceptibility make the hemin-binding Omp26 described by Bramanti and Holt clearly different from the HA2 domain (4, 30). We have therefore identified a second hemin-binding protein in *P. gingivalis*. Interestingly, a recent independent analysis of hemin binding by whole cells of *P. gingivalis* described two different affinities (64). Now we have demonstrated that hemin- or hemoglobin-binding activity is also released by *P. gingivalis* in batch cultures. It is not immediately clear what advantage *P. gingivalis* would gain by releasing heme-binding activity, but it may be speculated, considering the recovery of the separate HA2 domain from the outer membrane (43), that soluble HA2 might reassociate with other gingipain domains on the *P. gingivalis* cells after scavenging and binding to heme or hemoglobin. A specific association of the HA2 domain with an active catalytic domain may be required for removal of the heme moiety from hemoglobin.

Characterization of the binding between the rHA2 domain and porphyrins should allow design of efficient affinity ligands for purification of HA2 and allow structure-based design of inhibitors of heme or hemoglobin binding. Heme acquisition is considered to be fundamental to the growth of *P. gingivalis*, and intervention with specific agents to disrupt pathways for heme binding or uptake may allow the eventual control or prevention of periodontal disease.

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